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| (71) Applicant: HENRY M. JACKSON FOUNDATION FOR THE ADVANCEMENT OF MILITARY MEDECINE [US/US]; Suite 600, 1401 Rockville Pike, Rockville, MD 20852 (US). | | | |
| (72) Inventors: MCKEE, Marian, L.; 929 Holly Creek Drive, Great Falls, VA 27403 (US). O'BRIEN, Alison, D.; 5514 Charlotte Road, Bethesda, MD 20817 (US). WACHTEL, Marian, R.; 18705 Walkers Choice Road #2, Gaithersburg, MD 20879 (US). | | | |
| (74) Agents: GARRETT, Arthur, S. et al.; Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P., 1300 I Street, N.W., Washington, DC 20005-3315 (US). | | | |
| (54) Title: HISTIDINE-TAGGED INTIMIN AND METHODS OF USING INTIMIN TO STIMULATE AN IMMUNE RESPONSE AND AS AN ANTIGEN CARRIER WITH TARGETING CAPABILITY | | | |
| (57) Abstract <p>The present invention describes the isolation and purification of histidine-tagged functional portions of intimin (his-tagged intimin or his-intimin), a protein associated with the ability of certain strains of pathogenic bacteria to adhere to epithelial cells. The invention further describes the use of intimin as an antigen to promote a protective immune response. In addition, the invention describes the combination of intimin with one or more other antigens and administration of the combination to promote a protective immune response against intimin and the one or more antigens. One aspect of the invention is the administration of intimin to target specific epithelial cells to promote a protective immune response to intimin proteins. Additional aspects of the invention include the use of intimin or intimin combined with one or more antigens and administration of the combination to target gastrointestinal mucosa and stimulate an immune response. Additionally, the invention describes administration of the combination of intimin combined with drugs, to provide a means for targeted delivery of drugs to specific epithelial cells. Other aspects of the invention include the production of antibodies directed against his-intimin and methods of using such antibodies to provide passive immune protection, and in an assay system.</p> | | | |

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**HISTIDINE-TAGGED INTIMIN AND METHODS OF USING INTIMIN
TO STIMULATE AN IMMUNE RESPONSE AND
AS AN ANTIGEN CARRIER WITH TARGETING CAPABILITY**

GOVERNMENT INTEREST

The invention described herein may be manufactured, licensed and used for governmental purposes without the payment of any royalties to us thereon.

FIELD OF THE INVENTION

This invention relates to an isolated, functional bacterial protein, intimin, and its use as an antigen for protecting against infection by or transmission of bacteria expressing intimin-like proteins, such as certain pathogenic strains of *Escherichia coli*. The invention also relates to the use of intimin as a means for promoting an immune response to other antigens, and as a means of targeting delivery of antigens or medication to specific cells or locations within the body. The invention also relates to antibodies, both polyclonal and monoclonal, and their use in the treatment, diagnosis and prevention of infections by pathogenic *E. coli*.

BACKGROUND OF THE INVENTION

A virulent form of bloody diarrhea is caused by the Enterohemorrhagic *Escherichia coli* (EHEC). This pathogen is the most common infectious cause of bloody diarrhea (also called hemorrhagic colitis [HC]) in the United States (Centers for Disease Control and Prevention (executive summary). MMWR. 43(No.RR-5):1-18 (1994; Griffin, P.M. et al. Annals of Internal Med. 109:705 (1988). One serotype in particular,

O157:H7, is the most commonly isolated serotype of EHEC in the United States, and has been linked to a significant number of outbreaks of HC beginning in 1982 (Riley, L.W. et al. N.Eng.J. Med. 308:681 (1983)).

The primary mode of transmission of EHEC occurs through ingestion of contaminated food, particularly undercooked hamburger (Doyle, M.P. and Schoeni, J.L. Appl. Environ. Microbiol. 53:2394 (1987); Samadpour, M. et al. Appl. Environ. Microbiol. 60:1038 (1994)). Among people infected by EHEC, as many as 5 - 10% suffer a serious complication called Hemolytic Uremic Syndrome (HUS), a condition caused by the action of Shiga-like toxins that target and destroy cells lining blood vessels (endothelial cells), such as those present in the glomeruli of the kidney. (Johnson, W.M. et al. Lancet. i:76 (1983); O'Brien, A.D. et al. Lancet. i:702 (1983)). HUS can result in permanent kidney damage or even complete kidney failure.

Although EHEC can cause very serious illness even in healthy adults, young children in particular are at greater risk of dying or suffering permanent damage from the infection. Others for whom the infection can be particularly dangerous include the elderly and immuno-compromised. With the prevalence of EHEC in cattle and the subjective nature of differentiating between cooked and undercooked hamburger, a convenient stop at a fast food restaurant, or even a family barbecue, can result in family tragedy.

One key to the deadly nature of EHEC is the bacteria's ability to produce attaching/effacing (A/E) intestinal lesions in the colon, such as those demonstrated in gnotobiotic pigs (Tzipori, S. et al. Infect. Immun. 57:1142 (1989)). The A/E lesions

demonstrated in pigs are characterized by intimate bacterial adherence to the mucosal cells of the intestinal lining and dissolution of microvilli (McKee, M.L. et al. Infect. Immun. 63:3739 (1995); Tzipori, S. et al. Infect. Immun. 57:1142 (1989)). Similar lesions have been seen in human laryngeal epithelial (HEp-2)(ATCC # CCL23) cells in tissue culture (McKee, M.L. et al. Infect. Immun. 63:3739 (1995); Tzipori, S. et al. Infect. Immun. 57:1142 (1989)).

In 1990, Jerse et al. identified a chromosomal gene in a related diarrheagenic *E. coli* strain, Enteropathogenic *E. coli* (EPEC). That gene, designated *eae* (formerly known as *eaeA*), was found to be required for the bacterium to produce A/E lesions in tissue culture (Jerse, A.E. et al. Proc. Natl. Acad. Sci. USA. 87:7839 (1990)). The *eae* gene encoded a 94 kDa outer membrane protein (intimin), called *eae*, which is the intimin of EPEC. A similar protein was demonstrated to be present in an EHEC 0157:H7 strain (Jerse, A.E. and Kaper, J.B. Infect. Immun. 59:4302 (1991)).

Recently, investigators demonstrated that intimin is necessary for adherence of EHEC to human epithelial laryngeal (HEp-2) cells and human ileocecal epithelial (HCT-8) cells (ATCC # CCL244) (McKee, M.L. et al. Infect. Immun. 63:3739 (1995)) and for formation of A/E lesions in the piglet intestine (Donnenberg, M.S. et al. J. Clin. Invest. 92:1418 (1993); McKee, M.L. et al. Infect. Immun. 63:3739 (1995)). Although human studies with EHEC have not been conducted, as they are unethical and forbidden, the intimin protein found in EPEC is strongly associated with the production of diarrhea and fever in human volunteers (Donnenberg, M.S. et al. J. Clin. Invest. 92:1412 (1993); Levine, M.M. et al. J. Infect. Dis. 152:550 (1985)).

Human volunteers (10 out of 10) challenged with EPEC strain E2348/69 mounted a notable immune response to the 94 kDa protein after 28 days (Levine et al. J Infect. Dis. 152:550 (1985)). In these human trials the only volunteer (1 out of 10) who failed to develop diarrhea after ingestion of E2348/69 was the individual in this group who had detectable antibody to the 94 kDa protein intimin before challenge.

Two other bacterial species capable of inducing A/E lesions have been shown to contain the *eae* locus: *Hafnia alvei* (Albert, M.J. et al. J. Med. Microbiol. 37:310 (1992)) and *Citrobacter rodentium* (formerly known as *Citrobacter freundii* biotype 4280) (Schauer, D.B., and Falkow, S. Infect. Immun. 61:2486 (1993)). Although these bacteria are not generally associated with pathology in humans, they can cause significant disease in the animal species with which they are normally associated. For instance, *Citrobacter rodentium* is associated with gastrointestinal illness in mice. Mice often serve as control and test subjects in experiments. Costly and carefully controlled experiments can be jeopardized by an outbreak of this disease in an animal care facility. In addition, such bacterial species may become pathogenic to immuno-compromised patients, the young and the elderly.

The pathogens *Yersina enterocolitica* and *Yersina pseudotuberculosis* express a 103 kDa outer membrane protein (invasin) that allows bacterial penetration of cultured epithelial cells (Isberg, R.R. et al., Cell 60: 769 et seq. (1987)) and efficient penetration of the intestinal epithelium in vivo (Pepe, J.C. and Miller, V.L., Proc. Natl. Acad. Sci. USA 90:6473 et seq. (1993)). Invasin is also a member of the intimin protein family.

Animals, such as cows, infected with bacterial strains expressing intimin may become ill themselves, in addition to serving as a source of such infections to others. Eradicating or even limiting these animal reservoirs of intimin-expressing bacteria in animals with antibiotic therapy would be prohibitively expensive. In addition, not only is antibiotic treatment of the infections in humans or animals costly, but the antibiotics themselves are associated with side effects that can be dangerous. As with EHEC, those side effects can be especially dangerous to young children and the elderly. Consequently, the need exists for another means of reducing the seriousness of the infections or preventing them altogether through promotion of protective immune responses against bacteria expressing intimin.

A further need is for forms of immunization that are less time consuming, expensive and painful than immunization through injection of antigens. Yet another need is for the generation of protective immune responses in the specific tissues involved at the point of infection, most often the gastrointestinal mucosa.

Other organisms infecting gastrointestinal tissue, including, but not limited to *Salmonella sp.* and *Shigella sp.*, possess antigens against which an immune response could be generated. A need exists, however, for a means of targeting those antigens to gastrointestinal mucosa, in order to stimulate a mucosal immune response, as well as stimulating circulating antibodies.

Immuno-compromised individuals are less able to mount a protective immune response against pathogens, even with prior exposure to antigens associated with the pathogens. Thus a need exists to provide passive immune protection to

immunocompromised individuals exposed to the pathogens. A related need is the ability to identify whether an infection may be protected against by the presence of antibodies to intimin.

SUMMARY OF THE INVENTION

The present invention relates to an enriched protein comprising intimin or a portion of intimin that retains wild-type binding activity or that induces antibodies that block wild-type binding activity, as well as to a purified protein comprising intimin or a portion of intimin that retains wild-type binding activity or that induces antibodies that block wild-type binding activity. The invention also relates to a protein, comprising intimin, an intimin-like protein, or portion thereof, having a histidine tag. The invention further relates to the above-described proteins where the intimin, intimin-like protein, or portion thereof further comprises at least one antigen, at least one drug or a combination thereof chemically, physically or recombinantly conjugated with the intimin, intimin-like protein, or portion thereof.

Additionally, the present invention relates to a method for making purified intimin or a purified portion of intimin retaining binding activity, comprising expressing a protein comprising intimin having a histidine tag or a portion of intimin having a histidine tag, and removing the histidine tag from the intimin or portion of intimin. The invention further relates to a method for making a purified intimin-like protein or a portion thereof, comprising expressing a protein comprising an intimin-like protein, or a portion thereof,

having a histidine tag, and removing the histidine tag from the intimin-like protein, or portion thereof, before or after the purification.

The invention still further relates to a method for making enriched intimin or an enriched portion of intimin, comprising expressing a protein comprising intimin having a histidine tag or a portion of intimin having a histidine tag, enriching the intimin or portion of intimin, and optionally removing the histidine tag from the enriched intimin or enriched portion of intimin. The invention similarly relates to a method for making an enriched intimin-like protein or portion thereof, comprising expressing a protein comprising an intimin-like protein, or portion thereof, having a histidine tag, enriching the intimin-like protein or portion thereof, and optionally removing the histidine tag from the enriched intimin-like protein or portion thereof.

The invention also relates to a method of promoting a protective immune response against bacteria expressing intimin or intimin-like proteins, comprising administering to a patient intimin, an intimin-like protein, or portion thereof, wherein said portion retains wild-type binding activity or induces antibodies that block wild-type binding activity.

The invention additionally relates to a method of promoting a protective immune response against at least one antigen comprising administering to a patient a composition comprising at least one antigen chemically, physically or recombinantly conjugated to intimin, to an intimin-like protein, or to a portion thereof.

The invention further relates to a method of targeting the delivery of at least one antigen, at least one drug, or a combination thereof to epithelial cells, comprising

administering to a patient a composition comprising at least one antigen, at least one drug or a combination thereof, conjugated to intimin, to an intimin-like protein, or to a portion thereof having the ability to retain binding activity.

The invention still further relates to a method of providing passive immune protection comprising administering anti-intimin antibodies to a patient in need thereof.

The invention even further relates to a composition comprising anti-intimin antibodies, wherein the composition is free of other antibodies specific for an intimin-expressing host bacteria.

The invention even further yet relates to a method of preparing anti-intimin antibodies comprising expressing intimin having a histidine tag or a portion of intimin having a histidine tag, administering the intimin or portion of intimin to a patient, and recovering anti-intimin antibodies. The invention similarly relates to a method of preparing anti-intimin antibodies comprising expressing an intimin-like protein or portion thereof having a histidine tag, administering the intimin-like protein, or portion thereof, to a patient, and recovering anti-intimin antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts pEB313, a plasmid encoding RIHisEae. This plasmid encodes a histidine-tagged intimin that spans 900 out of 935 predicted C-terminal amino acids.

Fig. 2 depicts the predicted protein sequence of the complete EHEC 933 *eae* gene.

Fig. 3 depicts the DNA sequence from EHEC strain CL8, sequenced by Beebakhee, G. et al.

Fig. 4 depicts the DNA sequence from EHEC strain 933, sequenced by Yu and Kaper.

Fig. 5 depicts the 3144 bp fragment of *eae* produced by polymerase chain reaction amplification, in the region labeled *eae*.

Fig. 6 depicts pEB311, a plasmid encoding EHEC strain 86-24 *eae* (entire coding sequence) driven by the *lac* promoter.

Fig. 7 depicts pEB310, a plasmid encoding EHEC strain 86-24 *eae* (entire coding sequence) driven by the PT7 promoter.

Fig. 8 depicts histidine-tag expression plasmids (Qiagen Inc.).

Fig. 9 depicts the repressor plasmid (Qiagen Inc.) (multicopy).

Fig. 10 depicts pEB312, a plasmid encoding RVHindHis. This plasmid encodes a histidine-tagged intimin that spans 604 of 935 predicted amino acids.

Fig. 11 depicts the different fragments of *eae* cloned into his-tagged vectors, and the corresponding names of these plasmids.

Fig. 12 depicts the different C-terminal fragments of *eae* cloned into his-tagged vectors and the corresponding names of these plasmids.

Fig. 13 depicts the construction of an *eae* mutant, 86-24 *eae* Δ 10, by allelic exchange.

Fig. 14 depicts pEB290, a plasmid encoding most of the *eae* structural gene. The 3' 250 bp of *eae* are not encoded by pEB290.

Fig. 15 depicts pEB300, used to construct the deletion mutant; deleted for the 1275 bp internal Bcl I fragment of *eae*.

Fig. 16 depicts pAM450, a suicide vector for introduction of cloned genes into the bacterial chromosome.

Fig. 17 depicts pEB305i, a plasmid encoding the deleted *eae* gene in pAM450 vector for homologous recombination.

Fig. 18 depicts the cloning scheme for construction of a plasmid expressing N-His-IcsA-intimin-C.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to an enriched protein comprising intimin or a portion of intimin retaining wild-type binding activity or inducing antibodies that block wild-type binding activity, as well as to a purified protein comprising intimin or a portion of intimin retaining wild-type binding activity or inducing antibodies that block wild-type binding activity. The invention is also directed to a protein, comprising intimin, a portion of intimin, or an intimin-like protein, wherein the intimin, the portion of intimin, or intimin-like protein has a histidine tag. Preferably the histidine-tagged intimin, histidine-tagged portion of intimin, or histidine-tagged intimin-like protein is enriched or purified. It is also preferred that the proteins described above further comprise at least one antigen, at least one drug or a combination thereof chemically, physically or recombinantly conjugated with the intimin, the portion of intimin, or the intimin-like protein.

The invention additionally relates to a method for making purified intimin or a purified portion of intimin, comprising:

- expressing a protein comprising intimin or a portion of intimin, wherein the intimin or portion of intimin has a histidine tag,
- purifying the intimin or the portion of intimin, and
- removing the histidine tag from the intimin or portion of intimin before or after the purification.

The invention similarly relates to a method for making a purified intimin-like protein, comprising expressing a protein comprising an intimin-like protein having a histidine tag, purifying the intimin-like protein, and removing the histidine tag from the intimin-like protein before or after the purification.

The invention further relates to a method for making purified intimin or an enriched portion of intimin, comprising:

- expressing a protein comprising intimin having a histidine tag or a portion of intimin having a histidine tag,
- purifying the intimin or the portion of intimin, and
- removing the histidine tag from the purified intimin or enriched portion of intimin.

The invention similarly relates to a method for making an enriched intimin-like protein, comprising expressing a protein comprising an intimin-like protein having a histidine tag, enriching the intimin-like protein, and removing the histidine tag from the enriched intimin-like protein.

The invention even further relates to a method of promoting a protective immune response against bacteria expressing intimin or intimin-like proteins, comprising administering to a patient intimin, a portion of intimin retaining binding activity, or an intimin-like protein. Preferably the intimin, portion of intimin or intimin-like protein is purified or enriched. It is also preferred that the intimin, portion of intimin, or intimin-like protein has a histidine tag.

The invention still further relates to a method of promoting a protective immune response against at least one antigen comprising administering to a patient a composition comprising at least one antigen chemically, physically or recombinantly conjugated to intimin, to a portion of intimin, or to an intimin-like protein or portion thereof. Preferably the intimin, portion of intimin or intimin-like protein or portion thereof is purified or enriched. It is also preferred that the intimin, portion of intimin, or intimin-like protein or portion thereof has a histidine tag.

The invention even still further relates to a method of targeting the delivery of at least one antigen, at least one drug, or a combination thereof to epithelial cells, comprising administering to a patient a composition comprising at least one antigen, at least one drug or a combination thereof, conjugated to intimin, to a portion of intimin, or to an intimin-like protein or portion thereof. Preferably the intimin, portion of intimin or intimin-like protein or portion thereof is purified or enriched. It is also preferred that the intimin, portion of intimin, or intimin-like protein or portion thereof has a histidine tag.

The invention even further yet relates to a method of providing passive immune protection comprising administering anti-intimin antibodies to a patient in need thereof.

The invention additionally relates to a composition comprising anti-intimin antibodies, wherein the composition is free of other antibodies specific for an intimin-expressing host bacteria, which, as used herein, refers to any pathogenic or non-pathogenic bacteria expressing intimin, a portion of intimin, or an intimin-like protein or portion thereof encoded in the bacterial genome or in a plasmid. Preferably this host bacteria is EHEC. The antibodies produced by this method can be monoclonal antibodies or polyclonal antibodies. Preferably the antibodies produced by this method are affinity-purified.

The invention also relates to a method of preparing anti-intimin antibodies comprising expressing intimin having a histidine tag or a portion of intimin having a histidine tag, administering the intimin or portion of intimin to a patient, and recovering anti-intimin antibodies. This method can also include removing the histidine tag. Additionally, this method preferably comprises enriching intimin or said portion of intimin and more preferably comprises purifying the intimin or the portion of intimin.

The invention similarly relates to a method of preparing anti-intimin antibodies comprising expressing an intimin-like protein or portion thereof having a histidine tag, administering the intimin-like protein or portion thereof to a patient, and recovering anti-intimin antibodies. This method can also include removing the histidine tag. Additionally, this method preferably comprises enriching the intimin-like protein or portion thereof, and more preferably comprises purifying the intimin-like protein or portion thereof.

An object of the invention is to purify large quantities of intimin, preferably EHEC intimin, that retain the ability to bind epithelial cells. To achieve this object, a portion of *eae* was cloned into a Histidine-tag expression vector, expressed and purified. An additional object of this invention is to administer his-intimin as an antigen to elicit an immune response. The specific his-tagged intimin protein described above and those set forth in the examples below can be used for that purpose; however, those skilled in the art will recognize that a smaller fragment that retains binding function may also be desirable.

Those skilled in the art will also recognize that the size of the his-tagged intimin to be used may be varied according to the specific purpose for administering the intimin. For example, if the his-tagged intimin is to be conjugated with one or more antigens, a smaller fragment may be selected to enhance stability of the combined fusion product, although the use of a larger fragment is by no means precluded. The size or conformation of the other antigen and the location of its significant epitopes may indicate that a particular length of his-tagged intimin will bring the epitopes into close proximity to the mucosa. The desired size will also vary with the convenience of available restriction sites, in light of the materials and methods known to those of ordinary skill in the art. Consequently, the terms his-intimin and his-tagged intimin, as used herein, mean polypeptides containing at least 900 out of the 935 predicted C-terminal amino acids of intimin with a histidine tag (full-length intimin). The invention also includes smaller portions of the his-tagged protein that retain binding function.

Conjugates between his-tagged intimin and one or more antigens may be generated through recombinant technology to generate a fusion protein comprising intimin in a portion of intimin and an additional protein antigen. In addition, intimin may be chemically or physically conjugated to proteins, peptides, carbohydrates and other antigens by methods known to those skilled in the art. Methods of chemical conjugation are well known to those skilled in the art, and include, in part, coupling through available functional groups (such as amino, carboxyl, thio and aldehyde groups). See S.S. Wong, Chemistry of Protein Conjugate and Crosslinking CRC Press (1991); and Brenkeley et al. Brief Survey of Methods for Preparing Protein Conjugates With Dyes, Haptens and Cross-linking Agents, Bioconjugate Chemistry 3 #1 (Jan. 1992).

The retention of binding function means that the intimin, intimin-like proteins, and/or portions thereof retain the capacity to bind to epithelial cells or cell lines, such as HEp-2 and HCT-8. Such binding may be measured using any standard technique in the art. For example, the binding may be visualized as bacterial microcolony formation by the microcolony assay (Frankel et al., Infect. Immun. 62(5):1835-1842 (1994)), by FAS, (florescence actin staining), or both (McKee and O'Brien, Infect. Immun. 63(5):2070-2074 (1995), the disclosures of which is incorporated by reference herein). Additionally, binding may be measured *in vivo* as a function of bacterial virulence or pathogenicity, or by post-mortem histological examination. Examples of each of the above methods for determining binding function are detailed in Examples below, including the adherence assay described in Example IV.

One of the objects of the invention is to administer intimin or portions thereof, or intimin-like proteins, or portions thereof, to protect against illness or disease caused by EHEC or other pathogens having the capacity to bind epithelial cells through proteins having some degree of homology with the specific intimin expressed by EHEC. The object is achieved through stimulation of immune response directed against intimin, thereby blocking the capability of EHEC and related pathogens to adhere to epithelial cells. Consequently, the term immunization is used in the application.

The intimin, or portions of intimin, intimin-like proteins or portions thereof, are preferably capable of eliciting antibodies that block the binding of the corresponding wild-type protein. These antibodies may be directed against the binding site itself, or may be directed against any other portion of the linear polypeptide such that the antibodies block binding through steric hinderence or conformational changes. Such interference may be visualized, for example, by the loss of the wild-type binding pattern in the in vitro bacterial adherence assay of Example IV.

The degree of protection will vary with the degree of homology with intimin as well as the unique attributes of the patient, particularly as different species will be treated. The precise degree of protection is unimportant to quantitate in practicing the invention for any particular pathogen.

In addition to stimulating an immune response that permits a patient to avoid infection altogether, immunization as used herein also means decreasing the ability of the pathogens to colonize the gastrointestinal tract and decreasing the severity of an infection, as measured by any of the following indicators: reduced incidences of death,

HUS, or permanent kidney damage; decreased levels of toxins; reduced fluid loss; or other indicators of illness regularly used by those ordinarily skilled in the relevant art.

Unless specified otherwise, the uses and methods set forth herein are generally applicable to humans and animals. The term patient is used herein to mean both humans and animals, and animals are not limited to domesticated animals but also may include wildlife and laboratory animals as well.

Isolating and Purifying His-tagged Intimin

It has been shown that a His-intimin fusion, in which the N-terminal third of the molecule is deleted (RVHindHis), was capable of complementing adherence of a non-adherent EHEC *eae* mutant; i.e., restored binding capability to a strain of EHEC that lost its binding capability following genetic alterations that prevented expression of intimin. The pattern of adherence demonstrated in the restored binding was indistinguishable from that observed in wild-type strain 86-24 (McKee, M.L. and O'Brien, A.D. Infect. Immun. In press (1996)). Where measured by the microcolony assay, noted above, wild-type activity is identified as a punctate pattern with localized areas of intense staining.

Purification of the intimin protein, however, was difficult, in part because intimin is always associated with the outer membrane of EHEC. The majority of the overexpressed recombinant intimin remained associated with the bacterial membrane fraction, even after sonic disruption of the host bacterium and addition of mild detergent to the extraction buffer. The insolubility of the intimin protein, combined with the

abundance of other native *E. coli* proteins in the 97 kDa range, made purification of the native protein difficult.

Attempts to make an intimin-maltose binding protein fusion (MBP) also were unsuccessful because the predicted protein product had deletions and rearrangements. An attempt by other investigators had shown a construct of MBP fusions to the C-terminal 280 amino acids of intimin, but the fusion did not confer EHEC-like binding function. The diffuse pattern of adherence conferred by the MBP-intimin fusion protein was clearly different from the pattern of EHEC binding to HEp-2 cells (Frankel, G. et al. Infect. Immun. 62:1835 (1994)).

One possible explanation for the failure to obtain a functional MBP-intimin fusion larger than 280 amino acids is that overexpression of a piece of *eae* greater than the last 280 amino acids is unstable, and thus prone to rearrangements, i.e. it may be impossible to isolate that clone because it is lethal or deleterious to the cell when expressed. Alternatively, overexpression of a piece of MBP-intimin fusion larger than 280 amino acids could plug up the bacterial membrane, which would be lethal to the cells.

After trying unsuccessfully to purify intimin using MBP, a fusion was created using the QIAexpressionist Kit of QIAGEN, Inc., which involves attaching a histidine tag to the protein. The histidine tag is small, non-immunogenic, and binds tightly to a nickel affinity matrix, which facilitates purification of large quantities of material for further studies. In addition, the expression system permits one to maintain tight control of expression of the His fusion proteins to prevent any possible lethal effects of the

recombinant protein on the *E. coli* host strain as a result of overexpression of the protein. Example I describes the creation of such a fusion protein.

Example I

A. Construction of a plasmid, pEB313 (Figure 1), encoding His-tagged intimin encompassing 900 out of 935 predicted amino acids (Figure 2).

The *eae* gene is cloned from EHEC strain 86-24 (serotype 0157:H7), readily obtainable from Griffin, P.M. et al., Ann. Intern. Med. 109:705-712 (1988), or Phil Tarr (Children's Hospital and Medical Center, 4800 Sand Point Way NE, Seattle, WA. 98105, 206-526-2521.) The DNA is extracted according to standard chromosomal prep techniques (Wilson, K. in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) vol 1:2.4.1 - "Preparation of Genomic DNA from Bacteria").

The gene is cloned using the polymerase chain reaction (PCR), a standard technique in the art, using primers designed from a composite of the 2 known EHEC *eae* sequences. Two primers are constructed: Sn20- CGTTGTTAAGTCAATGGAAAC, a 5' primer, spanning bases 20-41 of the sequence from the strain CL8, which was sequenced by Beebakhee, G. et al. FEMS Microbiology. 91:63 (1992) (Figure 3); and MM2-TCTAGAGAGAAAACGTGAATGTTGTCTCT, a 3' primer, spanning bases 3061-3082 of the sequence from strain 933, sequenced by Yu, J. and Kaper, J.B. Mol. Microbiol. 6:411 (1992) (Figure 4). MM2 was further designed to include an XbaI site at the 3' end.

The PCR reactions are performed using the AmpliTaq Kit, according to the instructions of the manufacturer, Perkin Elmer. The PCR amplification produces a 3144

bp fragment encoding the entire *eae* open reading frame (ORF) (Figure 5, region designated *eae*) and includes 186 bp upstream. The PCR product is processed to create blunt ends and ligated into the *EcoRV* site of the vector pBRKS⁻ (Schmitt et al., J. Bacteriol. 176:368-377 (1994)).

The gene is cloned in both directions to allow transcription from either P_{lac} pEB311 (Fig. 6) and from P_{T7} pEB310 (Fig. 7) under the appropriate conditions. The plasmids are transformed into host strain XL1BlueF'Tn5 *lacI*_q, (available from QIAGEN, Inc., 9600 DeSoto Ave., Chatsworth, CA. 91311, 1-800-362-7737). The recombinants are maintained under the constitutive control of the *lac* repressor, because the previous failed attempts to clone *eae* suggested that overexpression of *eae* might be lethal to the host *E. coli* strain. The lower copy number of pBRKS⁻ vector, along with the control conferred by the *lac* repressor obviates these problems.

A his-tagged intimin plasmid is constructed by digesting pEB310 with *EcoRI*, filling in with Klenow fragment, digesting with *HindIII*, and isolating the resulting 2895 bp fragment. The DNA fragments are isolated using the GeneClean kit, Biol01 (1070 Joshua Way, Vista, CA. 92083, 1-800-424-61010). The his-tag expression plasmid pQE32 (Figure 8) (available from QIAGEN, Inc.) is digested with *SmaI* and *HindIII*. The 2895 bp fragment is then ligated to pQE32, creating pEB313 (Figure 1).

This plasmid, pEB313, encodes a his-tagged *eae* fragment of 101 kDa, called RIHisEae, which encodes 900 out of 935 predicted amino acids. This his-intimin fusion is constructed so that the N terminal 35 amino acids are deleted, to remove any

potential signal sequence. A signal sequence could target the fusion protein to the membrane or lead to cleavage of the His tag from the encoded intimin.

After the pEB313 construct is made, it is transformed into a lab strain of *E. coli* containing the *lac* repressor (*lacI_Q*), such as M15 pREP4 (repressor contained on the multicopy plasmid pREP4, supplied by QIAGEN, Inc.) (Figure 9) or XL1BlueF' Tn5/*lacI_Q* (repressor contained on the single copy F' plasmid, also available from QIAGEN, Inc.). The transformed *E. coli* express the his-intimin fusion protein encoded by pEB313. Purification of the protein is accomplished as set forth in Example III.

B. Construction of a plasmid, pHis-Inv1, encoding His-tagged *Yersinia pseudotuberculosis* invasin, and construction of a plasmid, pHis-Inv2, encoding His-tagged *Yersinia pseudotuberculosis* invasin with a deletion of the N-terminal 40 amino acids.

The plasmid pRI203 contains a 4.6 kb fragment of *Yersinia pseudotuberculosis* chromosomal DNA, including the *inv* gene and surrounding nucleotide sequences, and is readily obtainable from Dr. Ralph Isberg (Dept. Of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Mass. 02111; ref. R. R. Isberg, D. L. Voorhis, and S. Falkow. *Cell*. **50**:769 (1987)). The pRI203 DNA is extracted from the supplied bacterial strain with the use of a QIAGEN DNA extraction kit (QIAGEN, Chatsworth, CA.) according to the instructions of the manufacturer.

To construct pHis-Inv1, two primers, Inv1 (= 5' GTACGGATCCATGATGGTTTTCCAGCCAATCAGTGAG 3' and Inv3 (= 5' GTACGGTACCTTATATTGACAGCGCACAGAGCGGG 3') are used in a PCR reaction (as described above in part A) to amplify the desired *inv* sequences from pRI203. The

resulting 2960 bp *inv* fragment is digested with *Bam*HI and *Kpn*I, run on an agarose gel, excised with a razor, and purified using GeneClean (Bio101, LaJolla, CA.). The purified *inv* fragment is ligated into the His-tag QIAGEN vector containing the appropriate reading frame corresponding to the amplified *inv* sequence (pQE30, 31 or 32, QIAGEN, Chatsworth, CA), digested with *Bam*HI and *Kpn*I. The ligated plasmids are then transformed into DH5 α F'Tn5/*lac*I_q, and transformants checked for the presence of the appropriate size insert.

The plasmid pHis-Inv2 is constructed in the event that a signal sequence (and therefore the adjoining His tag) is cleaved from the protein expressed from pHis-Inv1. To construct pHis-Inv2, two primers, Inv2 (= 5' GTACGGATCCATATGTGGAATGTTTCATGGCTGGGG 3') and Inv3 (= 5' GTACGGTACCTTATATTGACAGCGCACAGAGCGGG 3') are used in a PCR reaction (as described in part A above) to amplify the desired *inv* sequences from pRI203. The resulting 2840 bp *inv* fragment is purified as above, and ligated into the His-tag QIAGEN vector as above, and transformed into a similar bacterial host strain.

Alternatively, similar plasmids are constructed by restriction enzyme digestion of pRI203, followed by ligation into the QIAGEN His-tag vector (pQE30, 31 or 32) containing the appropriate reading frame relative to the 5' end of the invasin fragment. The preceeding examples are meant to illustrate the construction of plasmids encoding histidine-tagged invasin from *Yersinia pseudotuberculosis*. One of ordinary skill in the art recognizes that analogous constructs, designed using alternative vectors and/or other intimin-like proteins can be constructed according to standard methods in the art.

One of ordinary skill in the art also recognizes that the above his-tagged invasins and other his-tagged intimin-like proteins may be applied to the methods disclosed elsewhere herein.

C. Construction of a plasmid (pEB312) (Figure 10) encoding His-tagged intimin encompassing 604 out of 935 predicted amino acids.

Digested plasmid pEB310 (obtained as described in part A) with *EcoRV* and *HindIII*, isolating the 1971 bp fragment, and ligating the fragment into pQE32 digested with *SmaI* and *HindIII*. Restriction enzymes, ligases, Klenow fragment used in protocols are from New England BioLabs (32 Tozer Rd., Beverly, MA. 01915-55991, 1-800-NEB-LABS) or Gibco BRL (P.O. Box 681 Grand Island, N.Y. 14072-0068, 1-800-828-8686). The resulting plasmid is designated pEB312.

The plasmid pEB312 encodes a his-tagged *eae* fragment called RVHindHis, which is about 65 kDa and encodes 604 out of 935 predicted amino acids. This construct contains the C-terminal two-thirds of the wild-type intimin protein.

As with the pEB310-expressed intimin, the fusion proteins remain primarily in the insoluble pellet after sonic disruption of the host *E. coli*. Therefore, urea and guanidine HCl are included in the purification protocol, which allows extraction of the fusion proteins from the insoluble pellet.

D. Construction of additional plasmids expressing different fragments of *eae*.

Each of these plasmids is tested to ensure that the protein fragment is capable of adhering to cultured cell lines using an adherence assay. (See example IV, below). In addition, the selection of the size of the intimin varies with whether the protein is

expressed alone and whether cross-immunity is desired with an intimin-like protein of known amino acid sequence. In addition to intimin expressed from EHEC and EPEC, examples of intimin-like proteins include, but are not limited to, intimin-like proteins of *Citrobacter rodentium*, *Hafnia alveii*, and the invasins of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*.

For instance, the larger 900 aa intimin is selected if there is heightened desire for cross-immunity with *Yersinia pseudotuberculosis*, because EHEC intimin shares 31% identical and 51% similar amino acids with the protein of *Yersinia pseudotuberculosis*, known as invasin (Yu, J. and Kaper, J.B. Mol. Microbiol. 6:411 (1992)). A greater degree of homology exists within the amino terminal two-thirds of the respective proteins.

Invasin is a 103 kDa outer membrane protein that allows bacterial penetration of cultured epithelial cells (Isberg, R.R. et al. Cell. 60:769 (1987)) and efficient penetration of the intestinal epithelium *in vivo* (Pepe, J.C. and V.L. Miller. Proc. Natl Acad. Sci. USA. 90:6473 (1993)). Cell adhesion receptors on the intestinal epithelium, which have been identified as integrins, bind invasin prior to bacterial internalization (Isberg, R.R. and J.M. Leong. Cell. 50:861 (1990)). The central portion of invasin is responsible for protein localization to the outer membrane, while the C-terminus is required for receptor binding (Isberg, R.R. Mol. Microbiol. 3:1449 (1989)).

Similarly, a larger 900 aa intimin is selected if there is heightened desire for cross-immunity with Enteropathogenic *Escherichia coli* (EPEC), *Citrobacter rodentium*, or *Hafnia alvei*. EPEC intimin shares 83% identity with EHEC intimin over the entire

length of the protein; the N-terminal 75% of the proteins share 94% identity, while the C-terminal 25% of the proteins share 49% identity (Yu, J. and Kaper, J.B. Mol. Microbiol. 6:411 (1992)). *Citrobacter rodentium* intimin shares 84% nucleotide identity to EHEC *eae* within the first 2106 bp, and 57% identity to the 3' 702 bp (Schauer, D.B. and Falkow, S. Infect. Immun. 61:2486 (1993)).

The plasmids that express different fragments of *eae* can be constructed using one of the following techniques: (1) use of a convenient restriction site within *eae*, for example, pEB313 digested with *SaI* and *HindIII*, isolation of the 1298 bp fragment, and ligation to pQE30 (QIAGEN, Inc.) digested with *SmaI* and *HindIII*. The resulting plasmid expresses the last 432 amino acids at the C terminus of *eae*; (2) deletion of the 5' or the 3' region of any desired fragment using nuclease BAL-31, Exonuclease III, or Mung bean nuclease (all available from New England BioLabs, 32 Tozer Rd., Beverly, MA 01915); (3) construction of plasmids with noncontiguous *eae* fragments, also using the above techniques; and (4) construction of plasmids encoding desired specific sequences with the use of PCR primers specifying the 5' and 3' ends of such sequences, as described in greater detail below.

With respect to the third technique, a His-tagged middle third intimin deletion mutant plasmid is constructed (pMW114). The plasmid pMW106 (described below) is transformed into the *dam* strain DM1F⁺Tn5/*acI*_O. DNA is made using the QIAGEN kit (QIAGEN, Inc.), and is digested with *BclI*. The DNA is run out on an agarose gel. The 5178 bp band is cut out, is purified using GeneClean (Bio 101), is ligated, and then transformed into DH5 α F⁺Tn5/*acI*_O (or other appropriate strain such as XL1Blue or

M15pREP4). Transformants are checked by restriction digestion of DNA. This description does not preclude the construction of other plasmids encoding non-contiguous *eae* sequences.

With respect to the fourth technique, PCR can be used to amplify specific fragments of *eae*; the fragments are isolated by restriction enzyme digestion and agarose gel electrophoresis, and ligated into the appropriate His-tag expression vector (i.e. p.QE30, 31 or 32; QIAGEN, Inc.).

For example, clones are constructed encoding various regions of *eae* (see Figures 11 and 12). The capacity of these clones to retain adherence function is assessed by either (1) transformation into the *eae* mutant, followed by adherence assays with HEp-2 cells (see Ex III, section C), or (2) addition of exogenous protein to bacteria (see Ex III, section D). It is important that the fragment selected retains full or close to full wild type binding activity. Alternatively, an acceptable clone from potentially any portion of the molecule is one which, encodes a polypeptide which can elicit antibodies which block the binding activity of wild-type intimin.

It is hypothesized that clones containing the highest binding activity will include the C-terminal third (the third third) of the protein, perhaps as little as 150 C-terminal amino acids. This hypothesis is supported, for example, by the findings disclosed by Frankel et al., Infection & Immunity, 62:1835 (1994), Frankel et al., Infection & Immunity, 63:4323 (1995), and Frankel et al., J. Biol. Chem., 271:20359 (1996). Proteins cannot be thought of as linear arrays of amino acids; rather they exist in a 3-dimensional structure. It is important to keep in mind that a single amino acid change

or a deletion of a portion of the protein can perturb this structure. Therefore, full cell binding activity may require the presence of additional non-contiguous sequences along with the third putative binding domain.

It is further hypothesized that clones containing high binding activity will include the two C-terminal Cys (encoded at bp 2780 and bp 3002, numbering ref; Beebakhee, G., J. DeAzavedo, and J. Brunton, FEMS Microbiology Letters 91:63 (1992)) for hypothesized disulfide bond formation and resulting loop formation. Additionally, it is hypothesized that clones containing high binding activity may require one or both aspartate(s) (encoded at bp 2819 and 2828, numbering ref. Beebakhee). This hypothesis is supported, for example, by analogy to invasins, as described in Leong, J.M., *Embo. J.* 14:422 (1995).

All clones are constructed in a similar manner. PCR primers are designed which specify the 5' and 3' region of the desired *eae* fragment. To facilitate cloning into pQE31, each 5' primer (MW1, MW3, MW5, MW7, MW8, MW9, MW10) contains a 5' *Bam*HI site, and each 3' primer (MW2, MW4, MW6, MW11, MW12) contains a 5' *Kpn*I site. Each PCR primer is designed so that the reading frame of the specified *eae* sequence is appropriate for insertion into pQE31. The following His-tagged constructs are cloned using the indicated PCR primers:

- (1) pMW101 - encodes the N-terminal third of *eae*; 27 kDa protein (PCR primers:
MW1 (5' PCR primer) = 5' GTACGGATCCGAATTCATTTGCAAATGGTG 3';
MW2 (3' PCR primer) = 5' GTACGGTACCTGATCAATGAAGACGTTATAG 3');

- (2) pMW102 - encodes the middle third of *eae*; 42 kDa protein (PCR primers MW3 (5' PCR primer) = 5' GTACGGATCCTGATCAGGATTTTCTGGTG 3'; MW4 (3' PCR primer) = 5' GTACGGTACCTGATCAAAAAATATAACCGC 3');
- (3) pMW103 - encodes the C-terminal third (282 amino acids) of *eae*; 32 kDa protein (PCR primers: MW5 (5' PCR primer) = 5' GTACGGATCCTGATCAAACCAAGGCCAGCATTAC 3'; MW6 (3' PCR primer) = 5' GTACGGTACCTTATTCTACACAAACCGCATAG 3');
- (4) pMW104 - encodes the N-terminal two thirds of *eae*; 69 kDa protein (PCR primers: MW1 and MW4);
- (5) p MW105 - encodes the C-terminal two thirds of *eae*; 73 kDa protein (PCR primers: MW3 and MW6);
- (6) pMW106 - encodes *eae* with a small N-terminal 35 amino acid deletion; 100 kDa protein (PCR primers: MW1 and MW6);
- (7) pMW108 - encodes the C-terminal 150 amino acids of *eae* (PCR primers: MW7 (5' PCR primer) = 5' GTACGGATCCACTGAAAGCAAGCGGTGGTGATg 3'; MW6);
- (8) pMW109 - encodes the C-terminal 140 amino acids of *eae* (PCR primers: MW8 (5' PCR primer) = 5' GTACGGATCCTTCATGGTATTCAGAAAATAC 3'; MW6);
- (9) pMW110 - encodes the C-terminal 130 amino acids of *eae* (PCR primers: MW9 (5' PCR primer) = 5' GTACGGATCCGACTGTCGATGCATCAGGGAAAG 3'; MW6);

- (10) pMW111 - encodes the C-terminal 120 amino acids of *eae* (PCR primers: MW10 (5' PCR primer) = 5' GTACGGATCCGAATGGTAAAGGCAGTGTCTG 3'; MW6);
- (11) pMW112 - encodes 120 amino acids of *eae* with the C-terminus, spanning bp #2560 - 2923, (numbering refers to *eae* sequence of strain CL8 ref. Beebakhee, G., J. DeAzavedo, and J. Brunton. FEMS Microbiology Letters 91:63)). (PCR primers: MW7; MW11 (3' PCR primer) = 5' GTACGGTACCTCCAGAACGCTGCTCACTAG 3');
- (12) pMW113 - encodes the C-terminal 282 amino acids of *eae*, Cys at bp 3002 changed to Ser with the use of the PCR primer MW12 (numbering refers to *eae* sequence of strain CL8 ref. Beebakhee, G., J. DeAzavedo, and J. Brunton. FEMS Microbiology Letters 91:63 (1992)) (PCR primers: MW5; MW12 (3' PCR primer) = 5' GTACGGTACCTTATTCTACAGAAACCGCATAG 3').

All clones are constructed by first diluting lyophilized primers to 10 μ M with dH₂O. Template DNA from strain XL1blue pEB310 (encoding the entire *eae* gene) is made using a QIAGEN prep (QIAGEN, Inc.), is linearized by digestion with a restriction enzyme that does not cut within or near the coding region, for example *HindIII*, and is quantitated using a spectrophotometer. PCR reactions are conducted by combining 10 μ l 10X Taq buffer (Perkin Elmer/Roche, Branchburg, N.J.), 10 μ l 2mM dNTP mix (Boehringer Mannheim, Indianapolis, IN.), 10 μ l 10 μ M 5' PCR primer, 10 μ l 10 μ M 3' PCR primer, 6 μ l 25 mM MgCl₂ (Perkin Elmer/Roche), 52 μ l dH₂O, and 1 μ l (1- 10 ng) linear

template DNA. Two drops of mineral oil are applied to the mixture, which is heated to 100°C for 5 minutes to denature the template. One μ l (5U) of AmpliTaq polymerase (Perkin Elmer/Roche) is added, and the PCR reactions are begun: 95°C/ 1min, 50°C/ 1 min, 72°C/ 3 min for 30 cycles, followed by 72°C/ 10 min, and holding at 4°C. After the PCR reactions are completed, the DNA is applied to a Wizard PCR clean-up kit (Promega, Madison, Wis.), and resuspended in 50 μ l TE buffer. PCR amplified DNA is digested with *Bam*HI and *Kpn*I, electrophoresed on an agarose gel, the appropriate size band cut out, and purified by Gene Clean (Bio101, LaJolla, CA.). Digested PCR fragments are then ligated into pQE31 digested with *Bam*HI and *Kpn*I, transformed into DH5 α F'Tn5/*ac*/_O (or other appropriate strain, such as M15pREP4 or XL1Blue), and transformants checked for the presence of the appropriate size insert.

With respect to any of the disclosed protein extraction, enrichment, and purification techniques, if it is necessary to later remove the Histidine tag from the purified protein, a protease cleavage site can be inserted between the 6XHis sequence and the N-(N-terminal tag) or C-terminus (C-terminal tag) of the protein. For example, Enterokinase recognizes the sequence "DDDK" (Asp₄-Lys), and cleaves after the lysine. A PCR primer encoding this sequence is designed and used to perform site-directed mutagenesis of the desired gene fragment. Alternatively, Carboxypeptidase A can be used for the removal of C-terminal His tags. This enzyme efficiently removes aromatic C-terminal residues (Hoculi, E. Chemische Industrie. 12:69 (1989)) until it encounters a basic residue, at which point removal is terminated. Additionally, PCR can be used to design a primer so that the protease site is encoded at the N- or C-

terminus of the protein encoded; or PCR can be used to design the vector including those sites, and the above-techniques can be used to clone into the aforementioned vector.

All fragments of intimin expressed from pEB312 or other constructs are purified using a protocol similar to the protocol detailed in Example II, for large scale purification of intimin. It is apparent that those of ordinary skill in the art may select additional restriction sites or modify the protocol while remaining within the scope and spirit of the invention.

Example II

Large scale enrichment of histidine-tagged Intimin

Growing Large-Scale Expression Cultures

Inoculate 20 ml LB (Luria-Bertaini) broth containing 100 µg/ml ampicillin and 40µg/ml kanamycin with a loopful of M15 pREP4 pEB313 (prepared as described in Example I, above). Grow overnight (15-18 h) at 37°C, shaking vigorously. Inoculate 1L of LB broth containing 100 µg/ml ampicillin and 40µg/ml kanamycin with 20 ml of the overnight culture. Grow culture at 37°C with vigorous shaking until the OD₆₀₀=0.7 - 0.9 (~3h). Add IPTG (isopropyl β-D-thiogalactopyranoside, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO. 63178, 1-800-325-3010) to a final concentration of 1mM (.476 g) and continue to grow culture for another 3h. Divide supernatant into 500 ml bottles (previously weighed) and centrifuge at 4000 X g for 10 minutes. Discard the supernatant, weigh cell pellet, and store at -70°C, or process immediately.

Thaw cells for 15 minutes, vortex and resuspend in Buffer A [6 M GuHCl, 0.1 M NaH_2PO_4 , 0.01 M Tris-HCl, pH 8.0] at 5 ml/g wet weight. Stir cells for 1 hour at room temperature. Centrifuge lysate at 10,000 X g for 15 min, collect supernatant. Add 5 ml of a 50% slurry of Ni-NTA resin (Ni-NTA slurry from QIAGEN, Inc), previously equilibrated with Buffer A. Stir at room temperature for 45 minutes, let the slurry settle, remove the supernatant, add 5 ml Buffer A, let the slurry settle, remove the supernatant, add 5 ml Buffer A, and load the resin into a column. The column is washed with 10 column volumes of Buffer A, followed by washes with Buffer B [8 M urea, 0.1 M NaH_2PO_4 , 0.01 M Tris-HCl, pH 8.0] until the $\text{OD}_{280} \leq 0.01$ (at least 5 column volumes). Wash the column with Buffer C [6M urea, 0.1M NaH_2PO_4 , 0.01 M Tris-HCl, pH 6.3] until the $\text{OD}_{280} \leq 0.01$. The protein is eluted with Buffer C plus 250 mM imidazole, collecting thirty 1 ml fractions.

Record the OD_{280} of each fraction. Pool aliquots of the purified protein containing similarly high OD_{280} readings into dialysis tubing (eg. Spectra/Por Cellulose Ester Membrane MW cut off=8000; Spectrum Medical Industries, 1100 Rankin Rd. Houston, TX. 77073-4716), and equilibrate in cold (4°C) Buffer C containing 6M urea (not 8M urea). Adjust the concentration of the aliquots to ≤ 1 mg/ml using a standard commercial protein quantitation kit (Bio-Rad Microassay, Bio-Rad Labs, 2000 Alfred Noble Dr., Hercules, CA. 94547, 1-800-4BIORAD), with BSA diluted in Buffer C (containing 6M urea) as the standard. Perform step dialysis of the protein in the cold (4°C) beginning with Buffer C (containing 6M urea) and reducing the molarity of the urea by whole number increments. Dialyze for one hour in each solution, ending in 1X



PBS. Analyze the protein by (10%) SDS-PAGE running ~2 μ l protein per well to verify protein size and quantity. The molecular weight of RIHisEae is 101 kDa.

Alternatively, add protein to dialysis tubing, dialyze straight into 1X PBS.

Quantitate the protein using a standard commercial protein quantitation kit (Pierce BCA Protein Assay Kit, Pierce, P.O. Box 117, Rockford, Ill. 61105), aliquot, and store at -20°C. As with the first alternative, analyze the protein by (10%) SDS-PAGE running ~2 μ l protein per well to verify protein size and quantity.

Upon enrichment of his-tagged intimin, the material derived is analyzed for level of purity by SDS-PAGE. A 10% SDS-PAGE gel is loaded with a 2 μ l sample of enriched his-tagged intimin and electrophoresed at 200 V for one hour. Molecular weight markers are included on the gel for size comparison. When the gel is stained with Colloidal Coomassie stain (Sigma, St. Louis, MO), the most prominent band appears at ~101kDa. Several other less prominent high molecular weights bands also appear. When the gel is stained with silver stain (BioRad, Richmond, CA) according to the instructions of the manufacturer, very slight high molecular weight bands appear, as well as several more prominent bands at low molecular weights, the most prominent band appearing around 29kDa. The enriched product preferably contains approximately 70-80% of the full-length (i.e., 900 out of 935 predicted amino acids) intimin. Preferably the enriched product contains no more than 25% contaminants (i.e., non-intimin related molecules), more preferably no more than 20% contaminants, still more preferably no more than 10% contaminants.

EXAMPLE III**Purification of Enriched Histidine-Tagged Intimin**

An enriched preparation of his-tagged intimin, generated as described in Example II above, is purified by techniques known to those skilled in the art, including, but not limited to, high performance liquid chromatography (HPLC), gel column chromatography, and SDS-PAGE.

With the SDS-PAGE method, an enriched preparation of his-tagged intimin is separated on a 10% polyacrylamide gel and visualized, for example, by staining an analytical lane with Colloidal Coomassie stain (Sigma, St. Louis, MO). The high molecular weight full-length intimin band can be excised from the preparative gel with a razor, and stored at 4°C prior to immunization. Less than full-length fragments of intimin, i.e. portions of intimin, and/or intimin conjugated to one or more antigens can similarly be excised from the gel.

Regardless of the method used to purify intimin, or portion thereof, the purified protein as used herein refers to a population of polypeptides consisting solely of intimin or portions of intimin, optionally tagged with histidine. It has been recognized in the art that the population of polypeptides expressed from a fragment of DNA containing only one open reading frame encoding intimin (or intimin-like proteins) can separate into multiple bands on an SDS-PAGE gel. McKee et al., *Infection & Immunity*, 64(6):2225-2233 (1996), Jerse et al., *Proc. Natl. Acad. Sci. USA* 87:7839-7843 (1990), and Isberg, *Cell* 50:769-778 (1987). Thus, purified intimin, or portions of intimin or intimin-like

proteins and intimin or intimin-like proteins conjugated with one or more antigens, may be visualized as multiple bands on an SDS-PAGE gel.

EXAMPLE IV

A. Adherence Assay.

Adherence of *E. coli* to either HEp-2 or HCT-8 cells is assessed by a modification of the method of Carvioto et al. Curr. Microbiol. 3: 95-99 (1979). Specifically, overlay semiconfluent monolayers of HEp-2 cells on glass coverslips in 24 well tissue culture dishes or in 8 well Permanox Chamber Slides (Nunc, Naperville, Ill.) with adherence assay medium (EMEM, or Eagle's Minimum Essential Medium supplemented with 0.4% sodium bicarbonate and 1% mannose) which contain 20 μ l/ml (v/v) of an overnight culture of the bacteria to be tested in LB broth.

Each inoculum contains $\geq 10^7$ bacteria (described below) which results in an approximate multiplicity of infection (MOI) of 100:1. The infected monolayers are incubated at 37° C in a 5% CO₂ atmosphere. After three hours, the medium, which contains the nonadherent bacteria, is aspirated and the monolayers washed once with sterile 10 mM phosphate buffered saline, pH 7.4 (PBS: sodium chloride, sodium phosphate dibasic, and potassium phosphate monobasic).

Fresh adherence assay medium is added to the cells with adherent bacteria, and the infected cells are then incubated for an additional 3 hours. The monolayers are then washed six times with PBS to remove nonadherent bacteria. Each wash is gently removed by aspiration in an attempt to avoid disturbing the monolayers. Each assay is

done ≥ 2 times and duplicate slides are prepared to permit both Giemsa and FITC-phalloidin (FAS) staining to visualize binding and associated sequelae.

For Giemsa staining, the HEp-2 cells and adherent bacteria are fixed with 70% (v/v) methanol (glass coverslips) or graded acetone washes (chamber slides) and stained with 1:10 Giemsa (Sigma) for 20 minutes. To assess the FAS phenotype, the FITC-Phalloidin (Sigma) staining procedure of Knutton et al. Infect. Immun. 57: 1290-1298 (1989) is used. Phalloidin is a mushroom phalloxin that specifically binds filamentous, not globular, actin. FITC-phalloidin-stained preparations are examined by both phase contrast and fluorescent microscopy using an Olympus model GHS microscope with a model BH2-RFL reflected light fluorescence attachment (Olympus Optical Co., Ltd., Tokyo, Japan).

Adherence assays with HCT-8 cells are done by the procedure described above for HEp-2 cells, but the bacteria are allowed to interact with the HCT-8 cells for 2.5 hours before the first wash and an additional 2.5 hours before terminating the assay. All assays with HCT-8 cells are carried out in 8 well permanox Chamber Slides.

**B. Construction of a Bacteria for Use in the Assay:
An EHEC *eae* mutant.**

To create an in-frame deletion in the chromosomal copy of the *eae* gene in a particular strain of EHEC, strain 86-24, the wild-type copy of the gene is replaced by double homologous recombination with an internally-deleted copy of *eae* (Figure 13). Plasmid pEB290 (Figure 14) encloses most of the *eae* structural gene and is constructed from a PCR product amplified from the 86-24 chromosome with primer

MM1 (MM1 = ATAACATGAGTACTCATGGTTG; starts at the second codon of the *eae* structural gene and includes a *ScaI* restriction site), in combination with primer MM2 (MM2 = TCTAGAGAGAAAACGTGAATGTTGTCTCT). The resultant 2,953 base pair fragment derived by PCR is digested with the *ScaI* and *XbaI* and ligated into pBluescript SK⁺ (Stratagene) that is restricted with *SmaI* and *XbaI*. DNA sequencing of the ends of the pEB 290 insert reveals that the 3' 250 base pairs are lost.

Plasmid pEB290 is transformed into *E. coli* strain GM119 [*dam*-6, *dcm* - 3, [Arraj, J.A. and Marinus, M.G. J. Bacteriol. 153:562-565 (1983)] to obtain unmethylated DNA which is sensitive to the restriction endonuclease *BclI*. Plasmid DNA is isolated (Maniatis, et al., Molecular cloning: a laboratory manual. Cold Spring Harbor (1982)) and restricted with *BclI* to remove an internal 1125 bp fragment from the gene. The resulting sticky ends are ligated to each other to create pEB300 (Figure 15).

The deleted *eae* gene is excised by digesting pEB300 with *XbaI* and *HindIII*, and the fragment containing the *eae* sequence is ligated into the *BamHI* site of the suicide vector, pAM450 (Figure 16) to form pEB305. Plasmid pAM450 is a derivative of pMAK705 (Hamilton et al., J. Bacteriol., 171:4617-4622 (1989)) with three features. First, it has a temperature sensitive (ts) origin of replication. Second, the plasmid carries the *sacB/R* locus from *Bacillus subtilis*, rendering the host strain sensitive to sucrose (Gay et al., J. Bacteriol 164:918-921 (1985)); Lepesant et al., Marburg. Mol. Gen. Genet. 118:135-160 (1972)). Third, the plasmid encodes ampicillin resistance. These features allow homologous recombination and positive selection for a second recombination event resulting in resolution and loss of vector sequences. The insertion

of the deleted *eae* gene (from pEB300) into the suicide vector (pAM450) results in the plasmid called pEB305 (Figure 17).

The suicide:*eae* construct, pEB305i, is transformed into wild type EHEC strain 86-24 by electroporation (Sizemore, et al., Microb. Pathog. 10:493-499 (1991)). Double recombinants that have been cured of the vector sequences are selected by growth on medium containing sucrose and then screened for ampicillin sensitivity (Blomfield et al., Mol. Microbiol., 5:1447-1457 (1991)). Transformants that have been cured of the suicide vector sequences are sucrose resistant, ampicillin sensitive, and able to grow equally well at 30° and 42° C. Deletion of the chromosomal *eae* sequences is confirmed by: (i) the reduced size of the *eae* fragment after PCR amplification with primers MM1 and MM2; (ii) Southern blot analysis of the mutated chromosomal DNA; (iii) loss of restriction sites within the deleted region of the *eae* gene; and (iv) the inability of an internal probe to recognize the mutated chromosome.

The resulting in-frame deletion mutant of EHEC strain 86-24 strain is designated 86-24*eae*Δ10. The mutation is confirmed to be in frame by *in vitro* transcription and translation analysis of the PCR-derived product from 86-24*eae*Δ10. A truncated protein product of the predicted size, about 68,000 Da, is identified by [³⁵S] methionine labeling of the translation product. The *eae* mutant strain is identical to wild type 86-24 in all characteristics, including: growth in LB broth, agglutination with O157 and H7 antisera, inability to ferment sorbitol, and growth on MacConkey agar at 37° C.

Those of ordinary skill in the art will recognize that other methods of creating strains of EHEC that are mutated in *eae* and do not retain binding ability are possible and may be substituted.

C. The role of *eae* in EHEC adherence *in vitro*.

The isogenic strains, 86-24, 86-24*eae*Δ10 and 86-24*eae*Δ10 carrying pEB310 are tested for adherence to HEp-2 and HCT-8 cells. Wild type 86-24 forms microcolonies when the bacteria interact with HEp-2 or HCT-8 cells. M.L. McKee & A.D. O'Brien, Infection & Immunity 63:2070 (1995). This localized adherence is FAS (fluorescence actin staining) positive which indicates the polymerization of F-actin at the site of bacterial attachment (i.e., the expected result). The mutant 86-24*eae*Δ10 is unable to adhere to HEp-2 cells. When *eae* is introduced into 86-24*eae*Δ10 on either pEB310 or pEB311, the LA /FAS (LA = localized adherence or microcolony formation) phenotype is fully restored, an observation which demonstrates that intimin alone complements the *eae* mutation. Since both of the clones complement the *eae* mutant, the native promoter for *eae* is present in the PCR amplified sequences.

D. Effect of Adding Exogenous His-intimin Fusion Proteins

The adherence assay also may be used to evaluate the effect of exogenously added His-intimin fusion proteins on the binding capability of 86-24*eae*Δ10 and the binding capability of wild-type strain 86-24. In this case, the purified His-intimin fusion proteins are added to the epithelial cell monolayers before addition of bacteria as indicated in each experiment.

HEp-2 cells are incubated with 20ng - 20µg of RIHisEae for 30 minutes prior to the addition of 86-24 to the monolayer. The infected monolayers are then washed extensively, stained with FITC-phalloidin, and observed microscopically. The fusions enhance binding wild type strain of 86-24 to HEp-2 cells. The size of the 86-24 microcolony as well as the total number of HEp-2 cells with adherent microcolonies increases as the concentration of RIHisEae increases. At high doses (20 µg), the fusion protein causes the HEp-2 cells to show aberrant appendages and processes. For this reason, 1-2 µg is the most preferred dose for further studies.

When added exogenously to HEp-2 cells, RIHisEae complements the HEp-2-cell binding defect (or restores binding capability) of 86-24 $eae\Delta 10$. The shorter fusion protein, RVHdHisEae, also complements for adherence. A similar amino terminal fusion of histidine residues to mouse dihydrofolate reductase (His-DHFR) does not enhance the adherence of 86-24. Moreover, the plasmids that encode the intimin fusion proteins, pEB312 and pEB313, are able to complement 86-24 $eae\Delta 10$ for attachment *in vitro*. Thus, such studies indicate that the proteins encoded by pEB312 and pEB313 are sufficient to confer adherence.

As noted above in Example I, the fusion proteins localize to the insoluble pellet fraction after sonic disruption of the host strains, indicating that these proteins are localized to the membrane. Plasmid pQE16, which encodes the His-DHFR fusion, does not complement 86-24 $eae\Delta 10$ (data not shown). That the irrelevant protein fusion with the histidine residues does not confer HEp-2 cell adherence on the *eae* mutant indicates that the histidine residues added to intimin are not responsible for the activity

observed for the exogenously added His-intimin fusions. The enhancement or complementation of EHEC binding to HEp-2 cells observable with exogenous RHisEae and RVHdHisEae indicates that intimin interacts with both the bacteria and the epithelial cell.

EXAMPLE V

The role of *eae in vivo* - Gnotobiotic piglet infection model

The role of intimin in intestinal colonization, A/E lesion formation, and EHEC-mediated colitis and diarrhea in the gnotobiotic piglet is evaluated by the method of Francis, et al. (Francis et al., Infect. Immun., 51:953-956 (1986)). Both pairs of piglets inoculated with the wild-type parent strain, 86-24 develop diarrhea and have edema in the mesentery of the spiral colon at necropsy.

Histologically, strain 86-24 primarily colonizes the cecum and spiral colon. Histologically and by culture, no evidence of bacterial dissemination to the liver, kidney, lung, or brain is detected. Intimate bacterial adherence and A/E lesions, as described by Staley (Staley et al., Vet. Pathol. 56:371-392 (1969)) and Moon (Moon et al., Infect. Immun., 41:1340-1351 (1983)) for EPEC, are evident by both light and EM examination of cecum and colon of piglets infected with 86-24. A/E lesions include the accumulation of electron-dense material at the site of attachment. In some areas, sloughed enterocyte fragments and microvilli with attached bacteria are noted in the gut lumen. In histologic sections of the cecum and spiral colon of piglets infected with 86-24, an inflammatory infiltrate is seen. Inflammation is characterized by scattered neutrophils in

the lamina propria and mild diffuse accumulation of serous fluid and perivascular lymphocytes and macrophages in the submucosa.

Both piglets inoculated with the mutant strain, 86-24 $\epsilon\epsilon\Delta$ 10 have formed feces at necropsy. Histologically and by EM examination, there is no evidence that strain 86-24 $\epsilon\epsilon\Delta$ 10 is able to colonize piglet intestine and cause the A/E lesion. The few bacteria seen by light and EM examination are in the mucus overlying the mucosal epithelium of the cecum and spiral colon. One of two piglets inoculated with 86-24 $\epsilon\epsilon\Delta$ 10 has slight mesocolonic edema, but no other gross or microscopic lesions are seen in either piglet. See McKee et al., *Infection and Immunity* 63: 3739 et seq. (1995) (incorporated herein by reference).

Piglets inoculated with 86-24 $\epsilon\epsilon\Delta$ 10(pEB310) have pasty feces and mesocolonic edema at necropsy. Strain 86-24 $\epsilon\epsilon\Delta$ 10(pEB310) intimately adheres to mucosal enterocytes and causes A/E lesions in the cecum and spiral colon. Histologically, perivascular lymphohistiocytic typhlocolitis, similar to that caused by wild type 86-24 is also seen.

Similar experiments are conducted in a colostrum-deprived newborn calf model, showing that intimin is necessary to provoke A/E lesions in the gut as well as to evoke *E. coli* O157:H7 strain 86-24-mediated diarrhea. (A.D. O'Brien, M.R. Wachtel, M.L. McKee, H.W. Moon, B.T. Bosworth, C. Neal Stewart, Jr., and E.A. Dean-Nystrom. "Intimin: Candidate for an *Escherichia coli* O157:H7 Anti-Transmission Vaccine". Abstract of the 32nd Joint Conference on Cholera and Related Diarrheal Diseases, Nagasaki, Japan, Nov 14-16, 1996, the disclosure of which is incorporated herein by

reference.) These experiments also demonstrate that by 2 days post-infection the numbers of infecting organisms in the lower bowel are significantly less in the animals fed the *eae* mutant or a non-pathogenic *E. coli* strain than in the calves fed the wild type or the *eae* mutant with the complementing clone.

EXAMPLE VI

Recognition of EHEC proteins by HC patient sera.

Convalescent immune sera tested from hemorrhagic colitis patients (kindly provided by T. Barrett at the Centers for Disease Control and Prevention, Atlanta, GA) react with P_{T7} -expressed intimin preparations (i.e., his-intimin expressed by pEB310 and pEB311) in a Western immunoblot. To decrease reactivity of the hemorrhagic colitis patients' sera with *E. coli* proteins in the expression system, sera samples are adsorbed with whole cell extracts of DH5 α transformed with pGP1-2 and pBRKS⁻ (the expression vector). After adsorption, the normal sera controls recognize only proteins in the ammonium sulfate concentrated fraction of the intimin preparations but no longer react with proteins expressed from pEB310 or the vector control. After adsorption, the HC patient sera still recognize many *E. coli* proteins, but the reaction with intimin remains strong.

EXAMPLE VII**Administration of His-intimin to patients**

The following example provides the administration of his-intimin to patients in order to stimulate a protective immune response. A protective immune response is one that elicits sufficient antibody to permit a patient to avoid infection, decrease the significance or severity of an infection, or decrease the ability of bacteria to colonize the gastrointestinal tract.

Methods of administration of his-intimin include, but are not limited to, injection (including, but not limited to, intraperitoneal, intravenous, subcutaneous, and intramuscular) of his-intimin directly into the patient to elicit an immune response, ingestion or by gavage of his-intimin alone or with food, and intra-nasal inoculation with his-intimin, which promotes binding of intimin to receptors of epithelial cells in the nasopharynx.

When the his-intimin is ingested, the protein is contained within a gel capsule, liposome, or attached to an inert substance to aid in passage of the inoculum through the stomach. As the fusion protein is acid stable, it also is ingested by itself or may be mixed into a food product. A preferred method of administration is in a fusion protein of his-intimin and SLT (Shiga-like toxin). A his-intimin-SLT fusion protein is bound to SYNSORB (SynSorb Biotech, Inc., 1204 Kensington Rd, N.W., Calgary, Alberta, Canada, T2N3P5), which has a receptor for SLT, via the SLT-receptor interaction. The SYNSORB construct is mixed with chocolate pudding and fed to children.

Purified RiHisEae (His-tagged Eae, 900/935 amino acids), as well as the third third portion of intimin (encoded by pMW103), are stable after incubation at pH 2.0 at 37° C for 24 hr. This indicates that a His-Eae fusion can pass through the stomach unharmed or undegraded. Moreover, in nature eae is expressed on the outer membrane of the bacterium and it still promotes intimate adherence after passing through the stomach, indicating its resistance to acidic environments.

Ingestion or intra-nasal inoculation stimulates local immunity, which thwarts future colonization by EHEC and EPEC. Cross-immunity through homology is stimulated to *Hafnia alvei* and *Citrobacter rodentium*, *Yersinia sp.* and other bacterial species having intimin-like proteins. Although it is not necessary to quantitate the degree of cross-immunity conferred by administration of intimin in order to benefit from a protective immune response to infection by bacteria other than EHEC that express intimin-related proteins, an assay for such protection is described in Example IX. The assay permits assessment of the efficacy of intimin antibodies on blocking interaction with epithelial cells by pathogens known to have intimin-like binding proteins.

In another embodiment, injection of his-intimin into cow udders leads to an immune response in the cow. Antibodies against the protein are present in the cow's milk. Calves that drink the milk are passively immunized until they can be actively immunized by the method of choice. Alternatively, his-intimin may be fed to cows or introduced into the cow's feed. The presence of his-intimin introduced in this way also stimulates an antibody response in the cows so that antibodies are produced and appear in the cows' milk.

Another embodiment involves the administration of nucleic acid vaccines. His-intimin is injected into a patient as naked *eae* DNA, or the DNA is delivered to the body by a carrier system such as retroviruses, adenoviruses, or other carriers known in the art. Following administration, the patient mounts an immune response against transiently expressed foreign antigens.

Currently nucleic acid vaccines, in general, are nearing clinical trials. This approach to vaccines involves delivering the DNA encoding the desired antigen into the host by inserting the gene into a nonreplicating plasmid vector (Marwick, C. JAMA 273:1403 (1995); reviewed in Vogel, F.R. and N. Sarver. Clin. Microbiol. Rev. 8:406 (1995)).

The first published demonstration of the protective efficacy of such a vaccine has shown that intramuscular injection of plasmid DNA encoding influenza A virus (A/PR/8/34) nucleoprotein (NP) elicited protective immune responses in BALB/c mice against a heterologous strain of influenza virus (A/HK/68) (Ulmer, J.B. et al. Science 259:1745 (1993)). Immunized animals had reduced virus titers in their lungs, decreased weight loss, and increased survival compared with challenged control mice. Both NP-specific cytotoxic T lymphocytes (CTL's) and NP antibodies were generated. The NP antibodies were ineffective at conferring protection, but the CTL's killed virus-infected cells and cells pulsed with the appropriated major histocompatibility complex class I-restricted peptide epitope.

Another study has shown that intramuscular injection of plasmid DNA encoding influenza virus A/PR/8/34 hemagglutinin resulted in the generation of neutralizing

antibodies that protected mice against a heterologous lethal influenza virus challenge (Montgomery, D.L. et al. DNA Cell Biol. 12:777 (1993)).

Practice of the invention by this method can be accomplished by reference to the aforementioned articles incorporated herein by reference, in particular Montgomery, D.L. et al. DNA Cell Biol. 12:777 (1993). The *eae* locus is described in Figure 5 according to restriction sites and according to its sequence in Fig. 3, for strain CL8, and its sequence in strain 933, shown in Figure 4.

EXAMPLE VIII

A. Conjugation of antigens from various pathogens to His-intimin to elicit an immune response against both *eae* and the conjugated antigen.

Antigens (Ag) and haptens from various pathogens are conjugated to a histidine-tagged intimin molecule. This fusion protein is used as an inoculum with intimin acting as the carrier to target binding to intestinal epithelial cells. This conjugate protein can be designed in any of the following configurations: N-His-intimin-Ag-C, N-Ag-intimin-His-C, N-His-Ag-intimin-C, N-intimin-Ag-His-C, N-intimin-His-Ag-C, or N-Ag-His-intimin-C.

The size of intimin varies with the size of the antigen that is to be fused, and the number of antigens to which the intimin is fused as would be recognized by those in the art. The variables to be considered in the design of such a fusion protein are: (1) foreign antigen; (2) size of intimin used, which can be of whatever size that retains binding function as described above; (3) fusion order N-C; and (4) method of

conjugation, such as genetic, as in cloning and expressing a fusion protein, and chemical, although additional methods are readily apparent to those ordinarily skilled in the art. (D.V. Goeddel, "Systems for Heterologous Gene Expression," Meth. Enzymol., Vol. 185, Academic Press, New York, 1990.; K. Itakura, "Expression in *E. coli* of a chemically synthesized gene for the hormone somatostatin," Science, 198: 1056-1063 (1977); and D.V. Goeddel et al., "Expression of chemically synthesized genes for human insulin," Proc. Natl. Acad. Sci. USA, 281: 544-548 (1979)).

Delivery of this coupled antigen occurs using the same mechanisms as that of a histidine-tagged intimin alone, as set forth above in Example VII.

Haptens and antigens may derive from but are not limited to bacteria, rickettsiae, fungi, viruses, parasites, drugs, or chemicals. They may include, for example, small molecules such as peptides, oligosaccharides, and toxins. Certain antimicrobial drugs and chemotherapeutic drugs having the capacity of being absorbed on the mucosal surface may also be coupled to intimin. The antigens and polysaccharides that may be coupled to intimin and administered to stimulate a protective immune response may include those shown below in Table 1.

TABLE 1

Antigens and/or polysaccharides from:

Bordetella pertussis
Borellia burgdorferi
Campylobacter sp., including *C. jejuni*
Candida albicans, other *Candida*
Chlamydi trachomatis and *pneumoniae* (TWAR)
Citrobacter rodentium
Clostridium sp., including *C. botulinum*, *C. difficile*, *C. perfringens*,
C. tetani, (including tetanus toxoid vaccine)
Coronaviruses
Corynebacterium diphtheriae, including diphtheria toxoid vaccine
Cryptococcus neoformans
Entamoeba histolytica
Escherichia coli sp. including
ETEC (enterotoxigenic *E. coli*),
EAggEC (enteroaggregative *E. coli*),
EPEC (enteropathogenic *E. coli*),
EHEC (enterohemorrhagic *E. coli*), EHEC SLT subunits or toxoid
EIEC (enteroinvasive *E. coli*),
UPEC (uropathogenic *E. coli*), including *E. coli* endotoxin,
J5 antigen (LPS, Lipid A, Gentabiose),
O polysaccharides (serotype specific)
EHEC
Haemophilus influenza, including *H. influenza* type b (polyribose
phosphate)
Hafnia alvei
Helicobacter pylori
Hepatitis A, B, C, and others
Human immunodeficiency virus I and II (GP120, GP41, GP160, p24,
and others)
Histoplasma capsulatum
Klebsiella species, including polysaccharides (serotype specific)
Legionella species, including *L. micdadei*, *L. pneumophila*
Listeria monocytogenes
Mycobacterium species, including *M. avium*, *M. kansasii*, *M. tuberculosis*
Mycoplasma
Neisseria species, including *N. gonorrhoeae*, *N. meningitidis*
(including serotype specific or protein antigens)
Nocardia asteroides
Plasmodium species

Pneumocystis carinii
 Polio virus
Pseudomonas aeruginosa, including serotype specific polysaccharides
 Rabies virus
 Rhinovirus
 Rickettsia
 Rotavirus
Salmonella sp., including *S. choleraesuis*, *S. enteritidis*, *S. typhi*,
 S. typhimurium
Shigella species, including *S. flexneri*, *S. sonnei*, *S. boydii*, *S. dysenteriae*
Staphylococcus sp., including *S. aureus*, polysaccharides from types 5 and 8
 (serotype specific and common protective antigens), *S. epidermidis*,
 serotype polysaccharide I, II, and III (and common protective
 antigens)
Streptococcus species, all serotypes including *S. pneumoniae* (all serotypes),
 S. pyogenes, including group A, group B (serotypes Ia, Ib, II, and III)
Treponema pallidum
 Varicella zoster
Vibrio cholerae
Yersinia species, including *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*

The sizes of his-intimin and portions of intimin, and intimin-like proteins and portions thereof, that may be conjugated to antigens appearing in Table 1 include RIHisEae (900/935 aa, EcoRI-HindIII fragment of pEB313) and RVHindHis (604/935 aa, EcoRV-HindIII fragment of pEB313), as set forth above in Example I. Those of ordinary skill in the art will recognize that additional fragments of varying lengths having adherence activity may be selected within the spirit and scope of the invention. The efficacy of the fragments considered for selection may be assessed according to the procedures described in Example IV.

B. Construction of a plasmid expressing N-His-IcsA-intimin-C.

Shigella flexneri causes bacillary dysentery in humans by invading epithelial cells of the colonic mucosa (Labrec et al. J. Bacteriol. 88:1503-1518, (1964)). A 120

kDa outer membrane protein, called IcsA, is necessary for intra- and intercellular spread of this organism (Bernardini et al. Proc. Natl. Acad. Sci. USA. 86:3867-3871, (1989); Lett et al. J. Bacteriol. 171:353-359, (1989)). An *iscA* mutant (SC560) was reasonably well tolerated by orally infected macaque monkeys and elicited protection against homologous challenge (Sansonetti et al. Vaccine 9:416-422, 1991).

The following protocol may be used (Figure 18):

Transform pEB313 into a *dam*⁻ host, such as DM1 (Gibco BRL, P.O. Box 68, Grand Island, N.Y. 14072, 1-800-828-6686). Digest pEB313/*Clal*/*Hind*III, isolate 1796 bp fragment (this fragment encodes the last 547 amino acids of intimin). Ligate into pBluescriptSK+/*Clal*/*Hind*III (pBluescriptSK+ available from Stratagene, 11011 N. Torrey Pines Rd., La Jolla, CA. 92037, 1-800-424-5444). Call this plasmid pEae1. Digest pHS3192 with *Ava*I, fill in the end with Klenow fragment, digest with *Clal*, isolate 2490 bp fragment [this fragment encodes 2923 bp or 974 aa's from base pair #706-3629; the ORF of *icsA* spans from bp#574-3880, this is 3306 bp and encodes 1102 aa's; reference for sequence of *icsA* is Lett et al., J. Bacteriol. 171:353 (1989)](pHS3192 available from P. Sansonetti (ref Bernardini, M.L. et al. Proc. Natl. Acad. Sci. USA. 86:3876 (1989)). Ligate the 2490 bp fragment into pEae1 digested with *Clal* and *Hinc*II, producing a plasmid called pEae2. Using these restriction enzymes, the reading frames of *icsA* and *eae* remain in frame. Digest pEae2 with *Xho*I and *Hind*III, isolate the 4286 bp fragment; ligate into pQE 32 (QIAGEN) digested with *Sma*I and *Hind*III. This ligation will maintain the proper reading frame of both genes with the promoter. The resulting plasmid is called plcsA-Eae.

Alternatively, one could fuse two genes in frame by cloning with PCR, followed by ligation into the appropriate pQE vector. This technique is well known to those of ordinary skill in the art.

C. Preparation of a conjugate vaccine using His-intimin as the protein carrier.

While any polysaccharide could be used, in this vaccine the capsular Vi polysaccharide of *Salmonella typhi* is used. Purify His-intimin as in Example II; this would be conjugated to Vi (purified from *S. typhi* according to established procedures (Szu et al. J. Exp. Med. 166:1510 (1987))). The conjugation will proceed using standard protein-polysaccharide conjugation technology well known to those in the art. Methods of conjugation are well known to those of ordinary skill in the art, and include the heteroligation techniques of Brunswick, M. et al., J. Immunol. 140:3364, 1988. See also Chemistry of Protein conjugates and Crosslinking, CRC Press, Boston (1991).

Techniques to conjugate moieties to primary or secondary carriers are well known to those skilled in the art, and include, in part, coupling through available functional groups (such as amino, carboxyl, thio and aldehyde groups). See S.S. Wong, Chemistry of Protein Conjugate and Crosslinking CRC Press (1991); and Brenkeley et al. Brief Survey of Methods for Preparing Protein Conjugates With Dyes, Haptens and Cross-linking Agents, Bioconjugate Chemistry 3 #1 (Jan. 1992).

A vaccine such as that described in this example would provide a prevention of diarrheal pathogens to include both those organisms that express intimin (or intimin-like proteins), as well as a diarrheal pathogen that expresses Vi.

Any combination of intimin plus other antigens from other diarrheal pathogens can be combined. In addition, if polysaccharides were used from organisms that produce other diseases, such as pneumococcal polysaccharides, the intimin-polysaccharide vaccine would be useful for prevention of multiple diseases. Delivery of a vaccine against respiratory pathogens will preferentially be done directly to the respiratory tract; ingested pathogens through ingestion.

EXAMPLE IX

Generation and testing of adherence-blocking anti-intimin antibodies: polyclonal and monoclonal

High titer polyclonal anti-intimin antisera are elicited upon intraperitoneal injection of RIHisEae into mice, rabbits, and goats. Testing of antibody titer and an antibody effectiveness assay are shown. The generation of monoclonal antibodies is also described.

A. Generation of Polyclonal Antibodies

Various techniques can be used to prepare antibodies against full-length intimin or various portions thereof in various animals. Several of these techniques are described below. As would be recognized by one skilled in the art, polyclonal antibodies can be generated from intimin and portions of intimin that are not his-tagged and from intimin-like proteins and portions thereof.

1. Generation of Mouse Anti-RIHisEae Polyclonal Antibodies

The technique of Harlow, E. and D. Lane (eds) Antibodies- a Laboratory Man. Cold Spring Harbor, New York (1988) may be followed. The general procedure is outlined herein. Take pre-bleeds of each mouse to be immunized: Bleed from the tail vein into an eppendorf tube. Incubate at 37° C for 30 min, stir gently with a sterile toothpick (to loosen the clot), store overnight at 4° C. In the morning, spin 10 min/10,000 rpm in the microfuge, and collect the serum (i.e., supernatant; red blood cells are the pellet). Store the serum at -20° C. The sera obtained will be used as a negative control after the mice are immunized.

Inject a BALB/c mouse intraperitoneally with 25 µg of RIHisEae (using Titremax adjuvant, according to the instructions of the manufacturer (CytRyx Corp., 154 Technology Pkwy., Norcross, GA. 30092, 800-345-2987). Wait 2 weeks, boost with an identical shot, wait 7 days and bleed from the tail vein into an eppendorf tube. Incubate at 37° C for 30 min, stir gently with a sterile toothpick (to loosen the clot), store overnight at 4° C. In the morning, spin 10 min/10,000 rpm in the microfuge, and collect the serum. Store the sera at -20° C.

2. Generation of Mouse anti-third third portion of Intimin polyclonal sera:

Mice are prebled by the tail vein as described above in Example IX, part A. The third third portion of intimin is enriched and dialyzed as described above in Example II. Mice are injected with the third third portion of intimin mixed with TitreMax adjuvant, as described in Example IX, part A. After 3 boosts, mice are bled via the retro-orbital sinus, and sera prepared in described Example IX, part A. Sera is tested

by Western blot analysis, as described for the goat polyclonal sera in section 4 below.

Sera is assayed for the capacity to block EHEC adherence to HEp-2 cells as described above in Example IX, part C.

3. Generation of Rabbit polyclonal anti-Intimin antibodies

Rabbit polyclonal sera is generated against the (1) first third, (2) second third, and (3) third third portions of intimin. Each specific sera is separately assayed in HEp-2 adherence assays for the capacity to block adherence of EHEC to HEp-2 cells.

Preparation of first third portion of intimin for rabbit immunization

Clone pMW101 is transformed into strain DH5 α F'lacI^q. Induction of protein expression and purification of the His-tagged intimin fragment over the Ni-NTA affinity resin is performed as described in the Qiagen manual that accompanies their QIAexpressionist Ni-NTA resin purification kit (Qiagen Inc., Chatsworth, CA). Eluted fractions are monitored for protein content by A₂₈₀ and by Bradford analysis, using a dye reagent from Bio-Rad (Hercules, CA). Peak fractions eluted from the Ni-NTA column (with 250mM imidazole) are electrophoresed on 15% polyacrylamide gels with SDS for analysis and purification. To visualize the proteins on the gels for analysis, both silver (Bio-Rad) and Coomassie Blue G-250 (Sigma) staining are used.

To purify the protein for immunization of animals to obtain antisera, the peak column fractions are run on preparative SDS polyacrylamide gels, and proteins are visualized with Copper stain (Bio-Rad). The band of protein corresponding to the intimin fragment is excised from the gel with a clean razor blade, and the gel slice is destained according to the instructions provided with the Copper stain reagent. Protein

is then eluted from the gel slice using an Electro-Eluter Model 422 (Bio-Rad) according to the manufacturer's instructions. The protein is then concentrated using a Centricon-10 concentrator from Amicon (Beverly, MA). The majority of the SDS in the eluted protein sample is removed by one of two methods. The first method involves addition of phosphate-buffered saline (PBS) to the protein sample, which causes precipitation of SDS. The majority of the protein does not precipitate, and the precipitate is not analyzed to determine what ions may also have precipitated. The SDS is pelleted by centrifugation; and the supernatant, which contains most of the protein and possibly some residual SDS, is removed and concentrated using a Centricon-10 concentrator from Amicon (Beverly, MA).

The second method for removal of SDS involves preparing a column of Extracti-Gel[®]D Detergent Removing Gel, purchased from Pierce (Rockford, IL). The Extracti-Gel[®]D Detergent Removing Gel is used according to the instructions of the manufacturer. The purified protein is concentrated as described above. Protein concentrations are determined by Bradford analysis using dye reagent purchased from Bio-Rad and also by running different volumes of purified protein on a gel adjacent to aliquots of varying amounts of the original column fractions to compare the amounts of proteins visually. Fractions of this purified protein are analyzed by SDS-PAGE using both silver and Coomassie staining.

Preparation of second third portion of intimin for rabbit immunization

Purification of the His-tagged middle third fragment of the intimin protein expressed from clone pMW102 is performed with the same methods used for the N-terminal third, with the following instructions. SDS-PAGE analysis is done using 12.5%

acrylamide gels. For gel-purification of the protein and electrolution, most of the preparative gels are stained with Copper stain as above; and one gel is stained with Coomassie brilliant blue dissolved in water as described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988). Much of the SDS in the electroeluted protein fractions is precipitated out by addition of PBS buffer. For concentration of the protein, Amicon Centricon-30 concentrators are used (Amicon).

Preparation of third third portion of intimin for rabbit immunization

The third third intimin protein is enriched and dialyzed as described above in Example II. One mg of protein is run by SDS-PAGE on four BioRad MiniProtean II gels. Protein is negatively stained with copper stain (BioRad, cat # 161-0470, Richmond, CA) according to the instructions of the manufacturer as follows: the gel is rinsed in dH₂O for 45 seconds, stained in 1X copper stain for 5 minutes, and rinsed in dH₂O for 3 minutes. The gel is visualized against a black background, and the ~37 kDa protein band is cut from the gel with a razor. Purified gel slices are then de-stained in buffer (25 mM Tris base, 192 mM glycine, 3X/ 10 min), wrapped in plastic wrap and stored at -20°C prior to immunization.

Immunization of Rabbits

New Zealand white female rabbits (5 to 6 lbs) are immunized separately with the antigens prepared as described above according to a schedule that could be readily determined by one skilled in the art. An example of such a schedule is as follows:

| DAY | PROCEDURE |
|-----|---|
| 0 | Prebleed/initial Inoculation, 100 µg Ag mixed with complete Freund's adjuvant |
| 14 | Boost, 50 µg mixed with incomplete Freund's adjuvant |
| 21 | Boost, 50 µg mixed with incomplete Freund's adjuvant |
| 35 | Test Bleed |
| 45 | Boost, 50 µg mixed with incomplete Freund's adjuvant |
| 56 | Test Bleed |

The route of injection can be subcutaneous and/or intramuscular at multiple sites. Sera derived from test bleeds is tested for specific recognition of the antigen by Western Blot analysis, as described for the goat polyclonal sera in section 4 below. When high titer recognition of the antigen is achieved, as recognizable by one skilled in the art, the rabbit is exsanguinated to recover the antibodies. The large volume sample of blood is verified for specific recognition of the antigen by Western Blot analysis.

Affinity Purification of Rabbit anti-intimin polyclonal sera by Western blot

Rabbit anti-intimin polyclonal sera is affinity purified to remove cross-reacting antibodies not specific for intimin or intimin-like proteins from the sera. (Harlowe, E. and D. Lane (eds) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988), p. 498 or S.H. Lillie and S.S. Brown. *Yeast*. 3:63 (1987)). RHisEae (0.250 mg) is electrophoresed by SDS-PAGE (size: BioRad MiniProtean II minigel, BioRad, Richmond, CA), transferred to nitrocellulose, and stained with Ponceau S (Sigma, St. Louis, MO.). A strip of nitrocellulose containing the full length His-intimin band (about 100 kDa) is excised with a razor, and the nitrocellulose strip containing the protein is incubated overnight at 4°C in 2% milk/TBS-0.2% Tween, shaking gently. The nitrocellulose strip is washed briefly in TBS-Tween, and placed in a container on top of

a piece of Parafilm (American National Can, Greenwich, Conn.). Rabbit sera is pipetted onto the mini-Western blot (as much volume as will fit, about 400-500 μ l), and wet paper towels are placed over the container, not touching the nitrocellulose strip, followed by plastic wrap. The blot is shaken gently for 5 hours, after which the sera (now called "depleted sera") is removed and saved for analysis. The strip is washed 3 times in PBS for 10 minutes, and glycine buffer (150 mM NaCl, pH 2.3-with HCl) is added (as much volume as will fit onto the strip) for 30 minutes. Affinity purified antibodies are pipetted off, and 1/10 volume Tris-HCl, pH 8.0 is added. Antibodies so recovered are then neutralized with 1N NaOH and tested by Western blot analysis as described below.

Affinity purification of rabbit anti-intimin polyclonal sera by antigen affinity column

Rabbit anti-intimin polyclonal sera is affinity purified to remove cross-reacting antibodies not specific for intimin from the sera. Antisera raised against intimin or various portions thereof is purified using an antigen affinity column using techniques known to those skilled in the art, such as those described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

The antigens (intimin or portions of intimin) are enriched as described above in Example II. Antigens may be further purified by electrophoresis on an acrylamide gel followed by electroelution from a gel slice containing the protein as described below in part 4. Other methods may be substituted for gel-purification and electroelution to further purify the protein after elution from the Ni-NTA resin. These methods may include, but are not limited to, ion-exchange column chromatography and gel filtration

chromatography. After purification, the intimin protein may need to be dialyzed into an appropriate buffer for coupling to activated beads to form the affinity resin for antisera purification.

Activated beads appropriate for coupling to the antigen are selected based on several properties: coupling reagent, binding group or matrix, ligand attachment, and stability of the final matrix (as listed in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988)). For example, the purified intimin (or portion of intimin) protein antigen is coupled to Affigel beads (Bio-Rad, Richmond, CA) according to the instructions of the manufacturer. A column of the activated beads coupled to the antigen is prepared and washed according to instructions of the manufacturer of the beads. The column is then washed according to the method described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

Ammonium sulfate precipitation is used to partially purify the sera in preparation for the affinity column. Ammonium sulfate precipitation, resuspension of the protein pellet in PBS, dialysis of the solution versus PBS, and centrifugation to clarify the solution are performed as described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

Antisera that has been partially purified by ammonium sulfate precipitation and dialysis versus PBS is passed over the antigen affinity column as described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988). The antisera may be passed over the column multiple times, as this may lead

to more complete binding of antibodies to the column. The column is then washed and the affinity-purified antibodies are eluted and dialyzed against PBS as described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

Adherence assays

Affinity purified polyclonal sera is assayed in HEp-2 cell adherence assays for the capacity to block bacterial binding to HEp-2 cells using the method described below in Example IX, part C.

4. Generation of Goat anti-RIHisEae polyclonal antibodies

Pre-bleeds are taken of potential goats to be immunized. Blood is collected from the jugular vein with indirect vacuum. Sera is separated from the whole blood, as described above in Example IX, section A, and tested by ELISA using RIHisEae as the adsorbent (as described in Example IX, section B, below for the ELISA and Example II above for the enrichment of RIHisEae), or by Western blot analysis as described below. The goat chosen for immunization has pre-immune sera with both (a) the lowest recognition of intimin by Western blot analysis and (b) the lowest titer against intimin by ELISA, and does not have the habit of jumping out of the pasture.

Western blot analysis of goat anti-RIHisEae polyclonal sera

a. Generation of whole cell lysates

Desired strains (for example: 86-24, 86-24 ϵ ae Δ 10, DH5 α , M15 pREP4 pEB313) are grown overnight in LB containing the appropriate antibiotics at 37° C, with shaking. Cells (4.5 ml) are pelleted in an eppendorf tube, and 500 μ l sonication buffer

(50 mM Na-phosphate pH 7.8, 300 mM NaCl) are added. Cells are sonicated in 15 second pulses on ice, aliquoted and frozen at -20°C.

b. Western blot analysis

Whole cell lysates generated as described above (2 - 5µl) or purified RIHisEae (2 µl) are run by SDS-PAGE, transferred to nitrocellulose, and used for Western blot analysis of goat sera. The sera (primary antibody) is typically diluted 1:500 or 1:1000 for this purpose. The secondary antibody used is swine anti-goat IgG conjugated to horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN), diluted 1:2000. Pre-bleeds of goat sera usually contain several cross-reactive bands that are removed later by affinity purification.

Preparation of purified RIHisEae (Antigen) for immunization into goat

One mg of RIHisEae, generated as described in Example II above, is run by preparative SDS-PAGE. A small analytical lane is stained with Colloidal Coomassie strain (Sigma, St. Louis, MO) and used for comparison to the rest of the preparative gel. The high molecular weight full-length intimin band (not stained, running at about 100 kDa) is excised from the preparative gel with a razor, and stored at 4°C prior to immunization.

Immunization of goats with antigen

Female goats (approximately one and a half years old, purebred Saanan or Saanan X LaMANCHA) are immunized separately with the antigens prepared as described above according to a schedule that could be readily determined by one skilled in the art. For example, the goat is given a primary immunization of 500 µg of

prepared RIHisEae mixed with Complete Freund's adjuvant. At two week intervals the goat is boosted with 250 µg Ag mixed with incomplete Freund's adjuvant. Test bleeds are begun after the goat has been immunized for a month, and continue until a high anti-intimin titer is reached, as defined by Western blot analysis, described above. When the sera recognizes intimin by Western blot, large blood samples are taken (500 mls, resulting in about 250 mls sera) per session, with two week intervals between large bleeds. Resulting large-volume sera samples are verified for recognition of intimin by Western blot analysis, as described above.

Affinity Purification of goat anti-intimin polyclonal sera by Western blot

Goat anti-intimin polyclonal sera is affinity purified to remove cross-reacting antibodies not specific for intimin from the sera. (Harlowe, E. and D. Lane (eds) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988), p. 498 or S.H. Lillie and S.S. Brown. *Yeast*. 3:63 (1987)). RIHisEae (0.250 mg) is electrophoresed by SDS-PAGE (size: BioRad MiniProtean II minigel, BioRad, Richmond, CA), transferred to nitrocellulose, and stained with Ponceau S (Sigma, St. Louis, MO.). A strip of nitrocellulose containing the full length His-intimin band (about 100 kDa) is excised with a razor, and the nitrocellulose strip containing the protein is incubated overnight at 4°C in 2% milk/TBS-0.2% Tween, shaking gently. The nitrocellulose strip is washed briefly in TBS-Tween, and placed in a container on top of a piece of Parafilm (American National Can, Greenwich, Conn.). Goat sera is pipetted onto the mini-Western blot (as much volume as will fit, about 400-500 µl), and wet paper towels are placed over the container, not touching the nitrocellulose strip,

followed by plastic wrap. The blot is shaken gently for 5 hours, after which the sera (now called "depleted sera") is removed and saved for analysis. The strip is washed 3 times in PBS for 10 minutes, and glycine buffer (150 mM NaCl, pH 2.3-with HCl) is added (as much volume as will fit onto the strip) for 30 minutes. Affinity purified antibodies are pipetted off, and 1/10 volume Tris-HCl, pH 8.0 is added. Antibodies are then neutralized with 1N NaOH and tested by Western blot analysis as described above.

Affinity purification of goat anti-intimin polyclonal sera by antigen affinity column

Goat anti-intimin polyclonal sera is affinity purified to remove cross-reacting antibodies not specific for intimin from the sera. Antisera raised against intimin or various portions thereof is purified using an antigen affinity column using techniques known to those skilled in the art, such as those described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

The antigens (intimin or portions of intimin) are enriched as described above in Example II. Antigens may be further purified by electrophoresis on an acrylamide gel followed by electroelution from a gel slice containing the protein as described below in part 4. Other methods may be substituted for gel-purification and electroelution to further purify the protein after elution from the Ni-NTA resin. These methods may include, but are not limited to, ion-exchange column chromatography and gel filtration chromatography. After purification, the intimin protein may need to be dialyzed into an appropriate buffer for coupling to activated beads to form the affinity resin for antisera purification.

Activated beads appropriate for coupling to the antigen are selected based on several properties: coupling reagent, binding group or matrix, ligand attachment, and stability of the final matrix (as listed in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988)). For example, the purified intimin (or portion of intimin) protein antigen is coupled to Affigel beads (Bio-Rad, Richmond, CA) according to the instructions of the manufacturer. A column of the activated beads coupled to the antigen is prepared and washed according to instructions of the manufacturer of the beads. The column is then washed according to the method described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

Ammonium sulfate precipitation is used to partially purify the sera in preparation for the affinity column. Ammonium sulfate precipitation, resuspension of the protein pellet in PBS, and dialysis of the solution versus PBS and centrifugation to clarify the solution is performed as described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

The antisera that has been partially purified by ammonium sulfate precipitation and dialysis versus PBS is passed over the antigen affinity column as described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988). The antisera may be passed over the column multiple times, as this may lead to more complete binding of antibodies to the column. The column is then washed and the affinity-purified antibodies are eluted and dialyzed against PBS as

described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

Adherence assays

Affinity purified polyclonal sera is assayed in HEp-2 cell adherence assays for the capacity to block bacterial binding to HEp-2 cells using the method described below in Example IX, part C.

B. ELISA to test titer of Antibodies

The technique of Harlow, E. and D. Lane (eds.) Antibodies- a Laboratory Manual. Cold Spring Harbor, New York (1988) may be followed. The general procedure is outlined below:

- (1) bind RIHisEae to plastic microtiter plates at 50 ng/well in PBS. Incubate 2h/RT (room temp) or overnight at 4° C.
- (2) wash plate 2X with PBS.
- (3) block wells with 100 µl blocking solution [3% bovine serum albumin (Sigma Chemical, St. Louis, MO.), 0.02% sodium azide (Sigma) in PBS - store stock at 4° C] for 1 - 2 h at RT.
- (4) wash plate 2X with PBS.
- (5) primary Ab = 50 µl test sera diluted in blocking solution for example, start with 1:50 and do eleven 1:2 dilutions, or start with 1:50 and do eleven 1:10 dilutions), incubate 2 h/RT.
- (6) wash 4X with PBS.

(7) secondary Ab = goat horseradish-conjugated anti-mouse (or other animal being tested) Ig, affinity purified (Boehringer Mannheim Corp., 9115 Hague Rd., P.O. Box 50414, Indianapolis, IN. 46250, 800-262-1640). Add secondary Ab diluted 1:500 in blocking solution without azide. Incubate 1h/RT.

(8) wash 4X with PBS.

(9) add 100 μ l TMB Peroxidase substrate to each well (prepared according to the instructions of the manufacturer, BioRad Labs, 3300 Regatta Blvd., Richmond, CA. 94804). Allow blue color to develop (no more than 10 min). Stop the reaction with 100 μ l H_2SO_4 . Read the plate at 450 nm.

A titer is defined as an absorbance value ≥ 0.2 units above that obtained for mouse pre-immune sera.

Anti-intimin antibodies may be administered to provide passive immune protection to a patient in need thereof. Moreover, anti-intimin antibodies obtained from animals may be used clinically in humans. In such cases, it is preferable to humanize the antibody. Such techniques are well known to those of ordinary skill in the art. See, for example, G. Winter et al., "Man-made antibodies," *Nature*, 349: 293-299 (1991); P.T. Jones et al., "Replacing the complementarity-determining regions in a human antibody with those from a mouse," *Nature*, 321: 522-525 (1986); P. Carter et al., "Humanization of an anti-p185^{HER2} antibody for human cancer therapy," *Proc. Natl. Acad. Sci. USA*, 89: 4285-4289 (1992). Such antibodies may be given to the sibling of an infected patient to reduce the risk of infection of the sibling.

C. Western blot analysis of anti-RIHisEae polyclonal sera

Polyclonal sera is assayed by Western blot analysis to verify recognition of intimin.

1. Generation of whole cell lysates

Desired strains (for example: 86-24, 86-24 θ ae Δ 10, DH5 α , M15 pREP4 pEB313) are grown overnight in LB containing the appropriate antibiotics at 37° C, with shaking. Cells (4.5 ml) are pelleted in an eppendorf tube, and 500 μ l sonication buffer (50 mM Na-phosphate pH 7.8, 300 mM NaCl) are added. Cells are sonicated in 15 second pulses on ice, aliquoted and frozen at -20°C.

2. Western blot analysis

Whole cell lysates generated as described above (2 - 5 μ l) or purified RIHisEae (2 μ l) are run by SDS-PAGE, transferred to nitrocellulose, and used for Western blot analysis of sera. The sera (primary antibody) is typically diluted 1:500 or 1:1000 for this purpose. The secondary antibody is specific for the animal that is the source of the primary antibody and is conjugated to horseradish peroxidase. Pre-bleeds of sera may contain several cross-reactive bands that are removed later by affinity purification.

D. Affinity Purification of anti-intimin polyclonal sera by Western blot

Anti-intimin polyclonal sera is affinity purified to remove cross-reacting antibodies not specific for intimin from the sera. (Harlowe, E. and D. Lane (eds) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988), p. 498 or S.H. Lillie and S.S. Brown. Yeast. 3:63 (1987)). RIHisEae (0.250 mg) is electrophoresed by SDS-PAGE (size: BioRad MiniProtean II minigel, BioRad,

Richmond, CA), transferred to nitrocellulose, and stained with Ponceau S (Sigma, St. Louis, MO.). A strip of nitrocellulose containing the full length His-intimin band (about 100 kDa) is excised with a razor, and the nitrocellulose strip containing the protein is incubated overnight at 4°C in 2% milk/TBS-0.2% Tween, shaking gently. The nitrocellulose strip is washed briefly in TBS-Tween, and placed in a container on top of a piece of Parafilm (American National Can, Greenwich, Conn.). Sera is pipetted onto the mini-Western blot (as much volume as will fit, about 400-500 µl), and wet paper towels are placed over the container, not touching the nitrocellulose strip, followed by plastic wrap. The blot is shaken gently for 5 hours, after which the sera (now called "depleted sera") is removed and saved for analysis. The strip is washed 3 times in PBS for 10 minutes, and glycine buffer (150 mM NaCl, pH 2.3-with HCl) is added (as much volume as will fit onto the strip) for 30 minutes. Affinity purified antibodies are pipetted off, and 1/10 volume Tris-HCl, pH 8.0 is added. Antibodies are then neutralized with 1N NaOH and tested by Western blot analysis as described above.

E. Affinity purification of anti-intimin polyclonal sera by antigen affinity column

Rabbit anti-intimin polyclonal sera is affinity purified to remove cross-reacting antibodies not specific for intimin from the sera. Antisera raised against intimin or various portions thereof is purified using an antigen affinity column using techniques known to those skilled in the art, such as those described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

The antigens (intimin or portions of intimin) are enriched as described above in Example II. Antigens may be further purified by electrophoresis on an acrylamide gel followed by electroelution from a gel slice containing the protein as described below in part 4. Other methods may be substituted for gel-purification and electroelution to further purify the protein after elution from the Ni-NTA resin. These methods may include, but are not limited to, ion-exchange column chromatography and gel filtration chromatography. After purification, the intimin protein may need to be dialyzed into an appropriate buffer for coupling to activated beads to form the affinity resin for antisera purification.

Activated beads appropriate for coupling to the antigen are selected based on several properties: coupling reagent, binding group or matrix, ligand attachment, and stability of the final matrix (as listed in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988)). For example, the purified intimin (or portion of intimin) protein antigen is coupled to Affigel beads (Bio-Rad, Richmond, CA) according to the instructions of the manufacturer. A column of the activated beads coupled to the antigen is prepared and washed according to instructions of the manufacturer of the beads. The column is then washed according to the method described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

Ammonium sulfate precipitation is used to partially purify the sera in preparation for the affinity column. Ammonium sulfate precipitation, resuspension of the protein pellet in PBS, and dialysis of the solution versus PBS and centrifugation to clarify the

solution is performed as described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

The antisera that has been partially purified by ammonium sulfate precipitation and dialysis versus PBS is passed over the antigen affinity column as described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988). The antisera may be passed over the column multiple times, as this may lead to more complete binding of antibodies to the column. The column is then washed and the affinity-purified antibodies are eluted and dialyzed against PBS as described in Harlow, E. and D. Lane (eds.) Antibodies - a Laboratory Manual. Cold Spring Harbor, New York (1988).

F. Assay for Blocking of Bacterial Binding by Antibodies to Intimin

To assess the effect of anti-intimin antibodies on EHEC adherence, mouse, rabbit, or goat anti-intimin antisera (or normal sera as controls) are added to EHEC bacteria suspended in adherence media, and the bacteria-antisera mixtures are incubated at 37°C for thirty minutes prior to infection of HEp-2 cells. Antisera are maintained in the adherence media throughout the assay. Adherence and related sequelae are microscopically observed using GIEMSA and FITC-phalloidin (FAS) staining as described above.

To assess the effect of anti-intimin antibodies on adherence of other bacteria having intimin-like proteins, mouse, rabbit, or goat anti-intimin antisera (or normal sera as controls) are added to EHEC bacteria suspended in adherence media, and the

bacteria-antisera mixtures are incubated at 37°C for thirty minutes prior to infection of HEp-2 cells.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

G. Raising Monoclonal Antibodies Specific for Intimin

Monoclonal antibodies directed against intimin are used to passively protect a patient against colonization by EHEC (or bacteria expressing intimin-like proteins).

Monoclonal antibodies are generated from mouse cells, and the specificity of these antibodies are changed for use in humans. G. Winter et al., "Man-made antibodies," *Nature*, 349: 293-299 (1991); P.T. Jones et al., "Replacing the complementarity-determining regions in a human antibody with those from a mouse," *Nature*, 321: 522-525 (1986); P. Carter et al., "Humanization of an anti-p185^{HER2} antibody for human cancer therapy," *Proc. Natl. Acad. Sci. USA*, 89: 4285-4289(1992). Monoclonal Abs represent a more "pure" antibody for administration to a patient.

1. Generation of anti-Eae monoclonal antibodies

Two examples of methods for generating anti-intimin monoclonal antibodies are described below.

a. Method 1**Generation of anti-Eae mAbs**

The procedure outlined in Harlow, E. and D. Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor, New York (1988) is followed with modifications. Nine week old female BALB/c (Harlan Sprague-Dawley, Indianapolis, IN) are used for the production of monoclonal antibodies. Prior to immunization, a serum sample is obtained from each mouse via the retro-orbital sinus. The whole blood is placed into a microfuge tube and allowed to cool at 4°C for between 4 and 16 hours. Serum is prepared by centrifugation of the whole blood at 1000 - 1200 X g for 15 minutes at 10-15°C. The serum is transferred to new microfuge tubes using a micropipettor and sterile pipets tips. The serum is stored at -20°C until use.

The antigen is obtained from SDS-PAGE gels of RIHisEae, obtained as described above in Example II. The high molecular weight intimin band is excised with a razor, as described above in Example IX, section A, part 4. One mg of RIHisEae is run onto four MiniProtean II gels (BioRad, Richmond, CA) for this purpose. Protein excised from the gels are made into a slurry in approximately 8 mls of phosphate buffered saline (PBS) using a mortar and pestle. On experimental day 0, a 0.8 ml portion of the slurry is mixed with 1.2 mls of complete Freund's adjuvant (CFA) and injected in 0.2 ml aliquots subcutaneously into each of four mice. A 0.5 ml portion of the slurry is mixed with 0.5 mls of RIBI T-700 adjuvant (RIBI Immunochem, Hamilton, Montana) and 0.2 mls is injected into each of four additional mice.

Mice receive booster injections on experimental days 21 and 42. The antigen is prepared as described above, with the exception that incomplete Freund's adjuvant (IFA) is used instead of CFA.

Serum samples are obtained as described above on experimental days 14, 35 and 49.

Serum samples are tested by immunoassay (as described below) to identify mice producing serum with the strongest response to *Eae*, as would be recognized to those skilled in the art. The reactivity of the serum samples is verified by Western blot analysis as described above in Example IX, section A, part 4. Three days prior to fusion (on experimental day 59), the mouse chosen for fusion is immunized with a 50% mixture of supernatant from the intimin slurry in PBS. A total of 0.1 mls of this slurry is injected intravenously via the tail vein.

Spleen cells from the chosen mouse are fused with SP2/0 myeloma cells (Cat # CRL1581 American Type Culture Collection, Rockville, MD 20850, 301-881-2600). A ratio of 10 spleen cells: 1 myeloma is used for the fusion. Fusion is accomplished by the use of polyethylene glycol (Cat # 783 641 Boehringer-Mannheim Corp., 9115 Hague Road, PO Box 50414, Indianapolis, IN 46250, 800-262-1640). Fused cells are distributed into 96-well tissue culture dishes for growth. Hybridomas are selected by growth of the cultures for 10 days in medium containing hypoxanthine, aminopterin and thymidine. Hybridomas secreting anti-intimin specific antibodies are identified from the 96-well tissue culture dishes by immunoassay as described below. Cultures positive for

antibodies reactive with *Eae* are expanded by transfer to 24-well dishes, retested for reactivity with *Eae* by immunoassay and cloned twice by limiting dilution.

Immunoassay (ELISA) of Mouse Polyclonal Anti-Intimin Serum and Hybridoma Supernatants (Anti-Intimin Monoclonal Antibodies)

A three ml portion of the intimin slurry used for immunization is centrifuged at approximately 1000 X g for 15 minutes at room temperature. A sample of the resulting, clarified supernatant is used to coat immunoassay plates. Briefly, the intimin-containing supernatant is diluted 1:300 in PBS and used as a coating antigen. Nunc Maxisorp Stripwells are coated with 100 µl/well of the diluted supernatant for 2-24 hours at room temperature.

Unbound material is washed from the wells with four washes of PBS containing 0.5% Tween-20 (PBS-T). For assays of serum samples, multiple dilutions of each sample are prepared in PBS-T and added to replicate wells. For assays of culture supernatants from 96-well dish cultures, each supernatant is diluted 1:2 in PBS-T and added to a single well. Supernatants from 24-well dish cultures are also diluted 1:2 in PBS-T and tested in duplicate. Assays of serum samples include a buffer control and a known polyclonal anti-intimin control. Assays of supernatants include a buffer control, medium control and a known polyclonal anti-intimin control.

Serum and supernatants are allowed to incubate in a draft-free environment at room temperature for 30-60 minutes on the intimin-coated wells and unbound antibodies and extraneous material (such as serum proteins) are washed from the wells

with four washes of PBS-T. Each well then receives 100 μ l of rabbit anti-mouse IgG (gamma specific)-HRP (Zymed, South San Francisco, CA), diluted 1:4000 in PBS-T.

The plates are again allowed to incubate in a draft-free environment at room temperature for 30-60 minutes. Each well then receives 100 μ l of one-component TMB substrate solution (Kirkegaard and Perry Labs, Gaithersburg, MD 20878, 301-948-7755). The reaction is allowed to proceed for 15 minutes in the dark and then stopped by the addition of 80 μ l/well of TMB stop reagent (Kirkegaard and Perry Labs, Gaithersburg, MD 20878, 301-948-7755).

b. Method 2

The procedure outlined in Harlow, E. and D. Lane, Antibodies, A Laboratory Manual. Cold Spring Harbor, New York (1988) is followed: Five 4- to 5-week old female BALB/cJ mice are prebled, and immunized intraperitoneally with 25 μ g RIHisEae suspended in 100 μ l of TiterMax. Mice are boosted twice in two week intervals, intraperitoneally with 25 μ g RIHisEae suspended in 100 μ l of TiterMax. Seven days after each boost, blood (~300 - 500 μ l) is collected from the tail vein. Sera are assayed for the presence of anti-RIHisEae antibody by ELISA (as described above).

Mice producing high titers of anti-RIHisEae antibodies are boosted both intravenously and intraperitoneally with 25 μ g of RIHisEae in 100 μ l of PBS, sacrificed three days later, and sera collected. Spleen cells are isolated and fused to Sp2/0-Ag mouse myeloma cells (ATCC #CRL1581) at a ratio of 10 spleen cells to 1 myeloma cell. Fused cells are distributed into microdilution plates, and culture supernatants are assayed by ELISA after 3-4 weeks of culture for RIHisEae antibodies. Cultures positive

for production of anti-RIHisEae antibodies are expanded and cloned twice by limiting dilution.

2. Determination of whether anti-RIHisEae mAbs recognize conformational or linear epitopes

Reactivities of the mAbs are compared by: 1) ELISA in which native RIHisEae is used as the adsorbent; and 2) immunoblot of RIHisEae denatured and separated by SDS-PAGE. Several pools of mAbs are obtained: 1) those that recognize only conformational epitopes and react positively by ELISA but not by immunoblot analysis; 2) those that recognize linear epitopes and react in both assays; and 3) those that recognize linear epitopes and react positively by immunoblot analysis, but not by ELISA. In addition, colony immunoblots of unlysed cells are done to determine if the mAbs recognize *Eae* expressed on the surface of the wild type strain 86-24.

3. Testing of anti-eae mAbs for capacity to block adherence of strain 86-24 to HEp-2 cells

Strain 86-24 is subjected to a qualitative adherence assay on HEp-2 cells and tested in parallel with bacteria that have been pre-incubated with various dilutions of anti-RIHisEae mAbs.

Selected adherence-blocking and conformational mAbs are subjected to isotype determination (Immunopure mAb Typing Kit, Pierce, Rockford, Ill.). Unique antibodies are then purified by affinity chromatography on a Protein G Sepharose column (Pharmacia, Piscataway, N.J.). The resulting affinity-purified mAbs are re-tested for capacity to block adherence of strain 86-24 to Hep-2 cells to ensure that the antibody remains functional after purification.

H. Use of polyclonal and monoclonal anti-intimin antibodies in diagnostic kits.

Diagnostic kits can be used to detect intimin-expressing bacteria, preferably EHEC. A general description of the preparation and use of such kits is provided in copending U.S. Application Serial No. 08/412,231, filed March 10, 1995, the disclosure of which is incorporated herein by reference.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

We claim:

1. An enriched protein comprising intimin or a portion of intimin, wherein said portion induces antibodies that block wild-type binding activity.
2. A purified protein comprising intimin or a portion of intimin, wherein said portion induces antibodies that block wild-type binding activity.
3. A protein, comprising intimin, an intimin-like protein, or a portion thereof, wherein said intimin, said intimin-like protein, or said portion thereof has a histidine tag.
4. A protein according to claim 3, wherein said intimin, said intimin-like protein, or portion thereof is enriched.
5. A protein according to claim 3, wherein said intimin, said intimin-like protein, or portion thereof is purified.
6. A protein according to claims 1, 2, 3, 4 or 5, wherein said intimin, intimin-like protein further, or portion thereof comprises at least one antigen, at least one drug or a combination thereof chemically, physically or recombinantly conjugated with said intimin, said intimin-like protein, or portion thereof.
7. A method for making purified intimin or a purified portion of intimin, comprising:
 - expressing a protein comprising intimin or a portion of intimin, wherein said intimin or portion of intimin has a histidine tag, and
 - purifying said intimin or said portion of intimin.
8. A method according to claim 7, further comprising removing said histidine tag from said intimin or portion of intimin before or after said purification.

9. A method for making a purified intimin-like protein or portion thereof, comprising expressing a protein comprising an intimin-like protein, or portion thereof, having a histidine tag, and purifying said intimin-like protein or portion thereof.

10. A method according to claim 9, further comprising removing said histidine tag from said intimin-like protein, or portion thereof, before or after said purification.

11. A method for making enriched intimin or an enriched portion of intimin, comprising:

- expressing a protein comprising intimin or a portion of intimin, wherein said intimin or portion of intimin has a histidine tag, and
- enriching said intimin or said portion of intimin.

12. A method according to claim 11, further comprising removing said histidine tag from said intimin or portion of intimin after said enrichment.

13. A method for making an enriched intimin-like protein or portion thereof, comprising expressing a protein comprising an intimin-like protein, or portion thereof, having a histidine tag, and enriching said intimin-like protein or portion thereof.

14. A method according to claim 13, further comprising removing said histidine tag from said enriched intimin-like protein or portion thereof.

15. A method of promoting a protective immune response against bacteria expressing intimin or intimin-like proteins, comprising administering to a patient intimin, an intimin-like protein, or a portion thereof which induces antibodies that block binding activity.

16. A method according to claim 15, wherein said intimin, intimin-like protein, or portion thereof is purified.

17. A method according to claim 15, wherein said intimin, intimin-like protein, or portion thereof is enriched.

18. A method according to claim 15, wherein said intimin, intimin-like protein, or portion thereof is histidine-tagged.

19. A method of promoting a protective immune response against at least one antigen comprising administering to a patient a composition comprising at least one antigen chemically, physically or recombinantly conjugated to intimin, to an intimin-like protein, or to a portion thereof, wherein said portion retains binding function.

20. A method according to claim 19, wherein said intimin, intimin-like protein, or portion thereof is purified.

21. A method according to claim 19, wherein said intimin, intimin-like protein, or portion thereof is enriched.

22. A method according to claim 19, wherein said intimin, intimin-like protein, or portion thereof is histidine-tagged.

23. A method of targeting the delivery of at least one antigen, at least one drug, or a combination thereof to epithelial cells, comprising administering to a patient a composition comprising at least one antigen, at least one drug or a combination thereof, conjugated to intimin, to an intimin-like protein, or to a portion thereof, wherein said portion retains binding function.

24. A method according to claim 23, wherein said intimin, intimin-like protein, or portion thereof is purified.

25. A method according to claim 23, wherein said intimin, intimin-like protein, or portion thereof is enriched.

26. A method according to claim 23, wherein said intimin, intimin-like protein, or portion thereof is histidine-tagged.

27. A method of providing passive immune protection comprising administering anti-intimin antibodies to a patient in need thereof.

28. A composition comprising anti-intimin antibodies, wherein the composition is free of other antibodies specific for an intimin-expressing host bacteria.

29. A composition according to claim 28, wherein said composition is free of other antibodies specific for EHEC.

30. A composition according to claim 28, wherein said antibodies are monoclonal antibodies.

31. A composition according to claim 28, wherein said antibodies are polyclonal antibodies.

32. A composition according to claim 28, wherein said antibodies are affinity-purified.

33. A method of preparing anti-intimin antibodies comprising:
- expressing intimin having a histidine tag or a portion of intimin having a histidine tag,
- administering said intimin or portion of intimin to a patient, and

- recovering anti-intimin antibodies.

34. A method according to claim 33, further comprising removing the histidine tag before said administration.

35. A method according to claim 33, further comprising enriching said intimin or said portion of intimin before said administration.

36. A method according to claim 35, further comprising removing the histidine tag after said enrichment.

37. A method according to claim 35, further comprising purifying said intimin or said portion of intimin before said administration.

38. A method according to claim 37, further comprising removing the histidine tag before or after said purification.

39. A method of preparing anti-intimin antibodies comprising:

- expressing an intimin-like protein, or portion thereof, having a histidine tag,
- administering said intimin-like protein, or portion thereof, to a patient, and
- recovering anti-intimin antibodies.

40. A method according to claim 39, further comprising removing the histidine tag before said administration.

41. A method according to claim 39, further comprising enriching said intimin-like protein or portion thereof before said administration.

42. A method according to claim 41, further comprising removing the histidine tag after said enrichment.



43. A method according to claim 41, further comprising purifying said intimin-like protein or portion thereof before said administration.

44. A method according to claim 43, further comprising removing the histidine tag before or after said purification.

45. An enriched protein comprising intimin or a portion of intimin, wherein said portion retains wild-type binding activity.

46. A purified protein comprising intimin or a portion of intimin, wherein said portion retains wild-type binding activity.

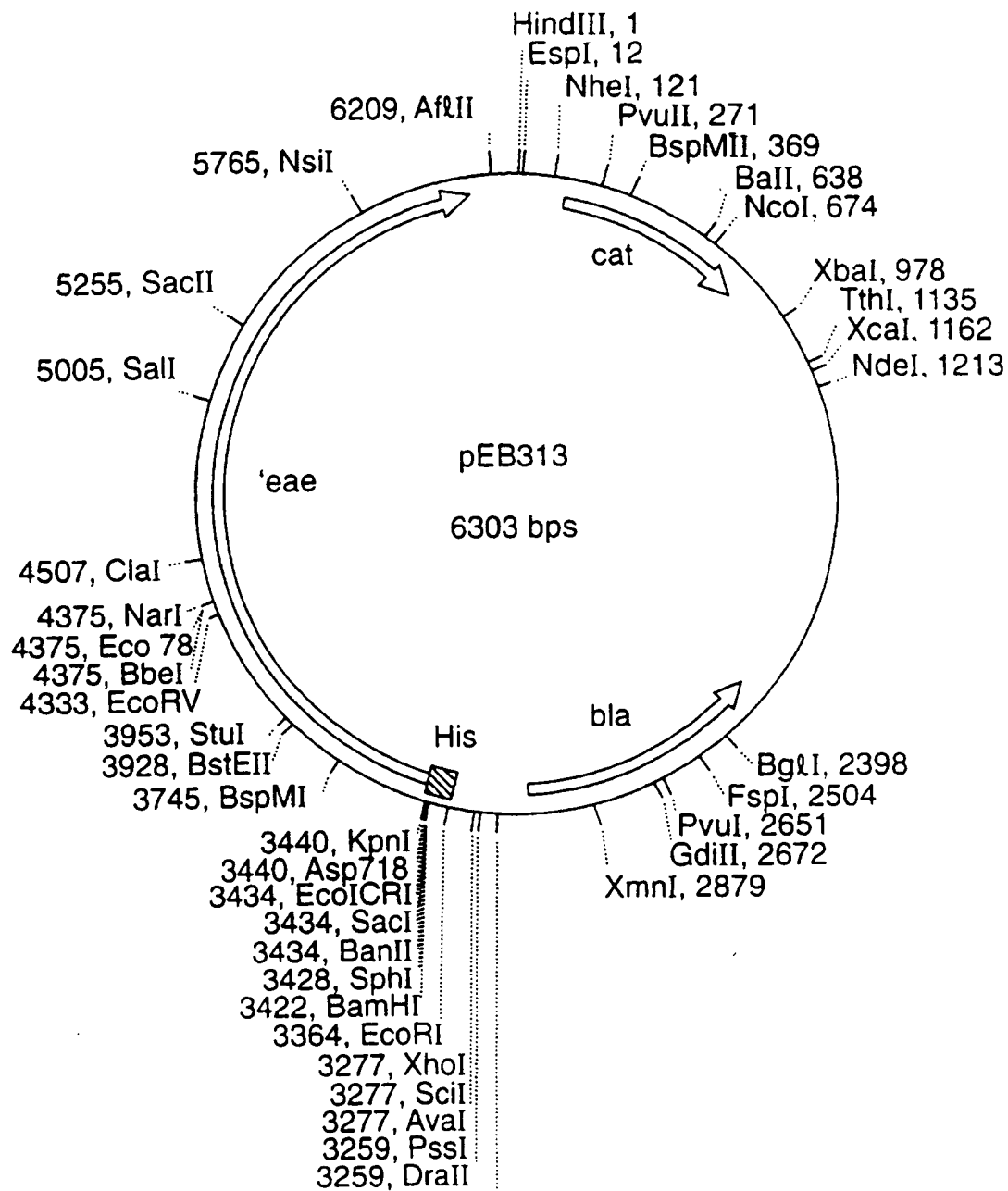
47. A method of promoting a protective immune response against bacteria expressing intimin or intimin-like proteins, comprising administering to a patient intimin, an intimin-like protein, or a portion thereof which retains binding activity.

48. A method according to claim 47, wherein said intimin, intimin-like protein, or portion thereof is purified.

49. A method according to claim 47, wherein said intimin, intimin-like protein, or portion thereof is enriched.

50. A method according to claim 47, wherein said intimin, intimin-like protein, or portion thereof is histidine-tagged.

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**FIG. 1****SUBSTITUTE SHEET (RULE 26)**

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| | | | | | |
|-----|------------|------------|------------|------------|------------|
| 1 | MITHGCTRT | RHKHKLKKT | IMLSAGLGLF | FYVNQNSFAN | GENYFKLGSD |
| 51 | SKLLTHDSYQ | NRLFYTLKTG | ETVADLSKSQ | DINLSTIWSL | NKHLYSSESE |
| 101 | MMKAAPGQQI | ILPLKKLPFE | YSALPLLGSA | PLVAAGGVAG | HTNKLTKMSP |
| 151 | DVTKSNMTDD | KALNYAAQQA | ASLGSQLQSR | SLNGDYAKDT | ALGIAGNQAS |
| 201 | SQLQAWLQHY | GTAEVNLQSG | DNFDGSSLDF | LLPFYDSEKM | LAFGQVGARY |
| 251 | IDSRTANLG | AGQRFLLPAN | MLGYNVFIQ | DFSGDNTRLG | IGGEYWRDYF |
| 301 | KSSVNGYFRM | RRWHESYHKK | DYDERPANGF | DIRFNGYLP | YPALGAK |
| 351 | EQYYGDNVAL | FNSDKLQSNP | GAATVGVNYT | PIPLVTMGID | YRHGTGNEND |
| 401 | LLYSMQFRYQ | FDKWSQQIE | PQYVNELR | SGSRYDLVQR | NNNIILEYK |
| 451 | QDILSLNIPH | DINGTEHSTQ | KIQLIVKSKY | GLDRIVWDDS | ALRSQGGQIQ |
| 501 | HSGSQSAQDY | QAILPAYVQG | GSNIYKVTAR | AYDRNGNSSN | NVQLTITVLS |
| 551 | NGQVVDQVGV | TDFTADKTS | KADNADTITY | TATVKKNGVA | QANVPVSFNI |
| 601 | VSGTATLGAN | SAKTDANGKA | TVTLKSSTPG | QVVVSAKTAE | MSSALNASAV |
| 651 | IFFDQTKASI | TEIKADKTTA | VANGKDAIKY | TVKVMKNGQP | VNNQSVTFST |
| 701 | NFGMFNGKSQ | TQATTGNDGR | ATITLTSSSA | GKATVSATVS | DGAEVKATEV |
| 751 | TFFDELKIDN | KVDIIIGNVR | GELPNIWLQY | GQFKLKASGG | DGTYSWYSEN |
| 801 | TSIATVDASG | KVTLNGKGSV | VIKATSGDKQ | TVSYTIKAPS | YMIKVDKQAY |
| 851 | YADAMSICKN | LLPSTQTVLS | DIYDSWGAAN | KYSHYSSMNS | ITAWIKQTSS |
| 901 | EQRSGVSSTY | NLITQNPLPG | VNVNTPNVYA | VCVE | |



FIG. 2

SUBSTITUTE SHEET (RULE 26)

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1   TCGAGAATGA AATAGAAGTC GTTGTTAAGT CAATGGAAAA CCTGTATTG GTATTACATA
61  ATCAGGGAAT AACATTAGAA AACGAACATA TGAAAAATAGA GGAAATCAGT TCAAGCGACA
121 ATAAACATTA TTACGCCGGA AGATAAAATC CGATCTATTA ATATAATTTA TTTCTCATTC
181 TAACTCATTG TGGTGGAGCC ATAACATGAT TACTCATGGT TGTTATACCC GGACCCGGCA
241 CAAGCATAAG CTAACAAAAA CATTGATTAT GCTTAGTGCT GGTTTAGGAT TGTTTTTTTA
301 TGTTAATCAG AATTCATTG CAAATGGTGA AAATTATTTT AAATTGGGTT CGGATTCAAA
361 ACTGTTAAC TATGATAGCT ATCAGAATCG CCTTTTTTAT ACGTTGAAAA CTGGTGAAAC
421 TGTTGCCGAT CTTTCTAAAT CGCAAGATAT TAATTTATCG ACGATTTGGT CGTTGAATAA
481 GCATTTATAC AGTTCTGAAA GCGAAATGAT GAAGGCCGCG CCTGGTCAGC AGATCATTTT
541 GCCACTCAA AAACCTCCCT TTGAATACAG TGCACTACCA CTTTTAGGTT CGGCACCTCT
601 TGTTGCTGCA GGTGGTGTG CTGGTCACAC GAATAAACTG ACTAAAAATG CCGCGACGT
661 GACCAAAAGC AACATGACCG ATGACAAGGC ATTAAATTAT GCGGCACAAC AGGCGCGGAG
721 TCTCGGTAGC CAGCTTCAGT CGCGATCTCT GAACGGCGAT TACGCGAAAG ATACCGCTCT
781 TGGTATCGCT GGTAACCAGG CTTGCTCACA GTTGCAGGCC TGGTTACAAC ATTATGGAAC
841 GGCAGAGGTT AATCTGCAGA GTGGTAATAA CTTGACGGT AGTTCAGTG ACTTCTTAT
901 ACCGTTCTAT GATTCCGAAA AAATGCTGGC ATTTGGTCAG GTCGGAGCGC GTTACATTGA
961 CTCCCGCTTT ACGGCAAAAT TAGGTGCGGG TCAGCGTTTT TCCCTTCTCG CAAACATGTT
1021 GGGCTATAAC GTCTTCATTG ATCAGGATTT TTCTGGTGAT AATACCCGTT TAGGTATTGG
1081 TGGCGAATAC TGGCGAGACT ATTTCAAAG TAGCGTTAAC GGCTATTTCC GCATGAGCGG
1141 CTGGCATGAG TCATACAATA AGAAAGACTA TGATGAGCGC CCAGCAAATG GCTTCGATAT
1201 CCGTTTTAAT GGCTATCTAC CGTCATATCC GGCATTAGGC GCCAAGCTGA TATATGAGCA
1261 GTATTATGGT GATAATGTTG CTTTGTTTAA TTCTGATAAG CTGCAGTCGA ATCCTGGTGC
1321 GCGGACCGTT GGTGTAAACT ATACTCCGAT TCCTCTGGTG ACGATGGGGA TCGATTACCG
1381 TCATGGTACG GGTAATGAAA ATGATCTCCT TTACTCAATG CAGTTCGGTT ATCAGTTTGA
1441 TAAATCGTGG TCTCAGCAA TTGAACCACA GTATGTAAAC GAGTTAAGAA CATTATCAGG
1501 CAGCCGTTAC GATCTGGTTC AGCGTAATAA CAATATTATT CTGGAGTACA AGAAGCAGGA
1561 TATTCTTTCT CTGAATATTC CGCATGATAT TAATGGTACT GAACACAGTA CGCAGAAGAT
1621 TCAGTTGATC GTTAAGAGCA AATACGCTCT GGATCGTATC GTCTGGGATG ATAGTGCAAT
1681 ACGCAGTCAG GCGGGTCAGA TTCAGCATAG CGGAAGCCAA AGCGCACAAG ACTACCAGGC
1741 TATTTTGCCT GCTTATGTGC AAGGTGGCAG CAATATTTAT AAAGTGACGG CTCGCGCCTA
1801 TGACCGTAAT GGCAATAGCT CTAACAATGT ACAGCTTACT ATTACCGTTC TGTGCAATGG
1861 TCAAGTTGTC GACCAGGTTG GGGTAACGGA CTTTACGGCG GATAAGACTT CGGCTAAAGC
1921 GGATAACGCC GATACCATTA CTTATACCGC GACGGTGAAG AAGAATGGGG TAGCTCAGGC
1981 TAATGTCCCT GTTTCATTTA ATATTGTTTC AGGAACTGCA ACTCTTGGGG CAAATAGTGC
2041 CAAAACGGAT GCTAACGGTA AGGCAACCGT AACGTTGAAG TCGAGTACGC CAGGACAGGT
2101 CGTCGTGTCT GCTAAAACCG CGGAGATGAC TTCAGCACTT AATGCCAGTG CGGTTATATT
2161 TTTTGATCAA ACCAAGGCCA GCATTACTGA GATTAAGGCT GATAAGACAA CTGCAGTAGC
2221 AAATGGTAAG GATGCTATTA AATATACTGT AAAAGTTATG AAAAACGGTC AGCCAGTTAA
2281 TAATCAATCC GTTACATTCT CAACAACTT TGGGATGTTT AACGGTAAGT CTCAAACGCA
2341 AGCAACCACG GGAATGATG GTCGTGCCAG GATACACTA ACTTCCAGTT CCGCCGTTAA
2401 AGCGACTGTT AGTCCGACAG TCAGTGATGG GGCTGAGGTT AAAGCGACTG AGGTCACTTT
2461 TTTTGATGAA CTGAAAATTG ACAACAAGGT TGATATTATT GGTAACAATG TCAAGAGGTC
2521 GATGTTGCCT AATATTTGGC TGCAATATGG TCAGTTTAAA CTGAAAGCAA GCGGTGGTGA
2581 TGGTACATAT TCATGGTATT CAGAAAATAC CAGTATCGCG ACTGTGATG CATCAGGGAA
2641 AGTCACTTTG AATGGTAAAG GCAGTGTCTG AATTAAAGCC ACATCTGGTG ATAAGCAAAC
2701 AGTAAGTTAC ACTATAAAG CACCGTCGTA TATGATAAAA GTGGATAAGC AAGCCTATT
2761 TGCTGATGCT ATGTCCATTT GCAAAAATTT ATTACCATCC ACACAGACGG TATTGTCAGA
2821 TATTTATGAC TCATGGGGGG CTGCAAATAA ATATAGCCAT TATAGTTCTA TGAAGTCAAT
2881 AACTGCTTGG ATTAACAGCA CATCTAGTGA GCAGCGTTCT GGAGTATCAA GCACCTATAA
2941 CCTAATAACA CAAAACCTC TTCCTGGGGT TAATGTAAAT ACTCCAAATG TCTATGCGGT
3001 TTGTGTAGAA TAATCCATA ACCACCCCGG CTAAATATG TATTGTTTTA GTCGGGGCAT
3061 AATTATTTCT TCTTAAGAAA TAACCTCTT ATAATCAAA CTACTACTGG TCTTTTATC
3121 TGCTTAATAG G

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FIG. 3**SUBSTITUTE SHEET (RULE 26)**

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|------|-------------|-------------|------------|------------|-------------|-------------|
| 1 | GGAAAGATAA | ATCCGATCTA | TTAATATAAT | TTATTTCTCA | TTCTAACTCA | TTGTGGTGGA |
| 61 | GCCATAACAT | GAGTACTCAT | GGTTGTTATA | CCCGGACCCG | GCACAAGCAT | AAGCTAAAAA |
| 121 | AAACATTGAT | TATGCTTAGT | GCTGGTTTAG | GATTGTTTTT | TTATGTTAAT | CAGAAATCAT |
| 181 | TTGCAAATGG | TGAAAATTAT | TTTAAATTGG | GTTCGGATTG | AAAACGTGTA | ACTCATGATA |
| 241 | GCTATCAGAA | TCGCCTTTTT | TATACGTTGA | AAACTGGTGA | AACTGTTGCC | GATCTTTCTA |
| 301 | AATCGCAAGA | TATTAATTTA | TCGACGATTT | GGTCGTTGAA | TAAGCATTTA | TACACTTCTG |
| 361 | AAAGCGAAAT | GATGAAGGCC | GCGCCTGGTC | AGCAGATCAT | TTTGCCACTC | AAAAAACTTC |
| 421 | CCTTTGAATA | CAGTGCACCT | CCACTTTTAG | GTTCGGCACC | TCCTGTTGCT | GCAGGTGGTG |
| 481 | TTGCTGGTCA | CACGAATAAA | CTGACTAAAA | TGTCCCCGGA | CGTGACCAAA | AGCAACATGA |
| 541 | CCGATGACAA | GGCATTAAT | TATGCGGCAC | AACAGGCGGC | GAGTCTCGGT | AGCCAGCTTC |
| 601 | AGTCGCGATC | TCTGAACGGC | GATTACGCGA | AAGATACCGC | TCCTGGTATC | GCTGGTAACC |
| 661 | AGGCTTCGTC | ACAGTTGCAG | GCCTGGTTAC | AACATTATGG | AACGGCAGAG | GTTAATCTGC |
| 721 | AGAGTGGTGA | TAACTTTGAC | GGTAGTTTAC | TGGACTTCTT | ATTACCGTTC | TATGATTCCG |
| 781 | AAAAATGCT | GGCATTTGGT | CAGGTCGGAG | CGCGTTACAT | TGACTCCCGC | TTTACGGCAA |
| 841 | ATTTAGGTGC | GGGTCAGCGT | TTTTTCCTTC | CTGCAACAT | GTTGGGCTAT | AACGCTTCA |
| 901 | TTGATCAGGA | TTTTTCTGGT | GATAATACCC | GTTTAGGTAT | TGGTGGCGAA | TACTGGCGAG |
| 961 | ACTATTTCAA | AAGTAGCGTT | AACGGCTATT | TCCGCATGAG | GCGCTGGCAT | GAGTCATACC |
| 1021 | ATAAGAAAGA | CTATGATGAG | CGCCCAGCAA | ATGGCTTCGA | TATCCGTTTT | AATGGCTATC |
| 1081 | TACCGTCATA | TCCGGCATT | GGCGCCAAGC | TGATATATGA | GCAGTATTAT | GGTGATAATG |
| 1141 | TTGCTTTGTT | TAATCTGAT | AAGCTGCAGT | CGAATCCTGG | TGCGGCGACC | GTTGGTGTA |
| 1201 | ACTATACTCC | GATTCCTCTG | GTGACGATGG | GGATCGATTA | CCGTCATGGT | ACGGGTAATG |
| 1261 | AAAATGATCT | CCTTTACTCA | ATGCAGTTCC | GTTATCAGTT | TGATAAATCG | TGGTCTCAGC |
| 1321 | AAATTGAACC | ACAGTATGTT | AACGAGTTAA | GAACATTATC | AGGCAGCCGT | TACGATCTGG |
| 1381 | TTCAGCGTAA | TAACAATATT | ATTCTGGAGT | ACAAGAAGCA | GGATATTCTT | TCTCTGAATA |
| 1441 | TTCCGCATGA | TATTAATGGT | ACTGAACACA | GTACGCAGAA | GATTCAAGTTG | ATCGTTAAGA |
| 1501 | GCAAATACGG | TCTGGATCGT | ATCGTCTGGG | ATGATAGTGC | ATTACGCGAGT | CAGGGCGGTC |
| 1561 | AGATTACAGCA | TAGCGGAAGC | CAAAGCGCAC | AAGACTACCA | GGCTATTTTG | CCTGCTTATG |
| 1621 | TGCAAGGTGG | CAGCAATATT | TATAAAGTGA | CGGCTCGCGC | CTATGACCGT | AATGGCAATA |
| 1681 | GCTCTAACAA | TGTACAGCTT | ACTATTACCG | TTCTGTCGAA | TGGTCAAGTT | GTGACACAGG |
| 1741 | TTGGGGTAAC | GGACTTTACG | GCGGATAAGA | CTTCGGCTAA | AGCGGATAAC | GCCGATACCA |
| 1801 | TTACTTATAC | CGCGACGGTG | AAAAAGAATG | GGGTAGCTCA | GGCTAATGTC | CCTGTTTCAT |
| 1861 | TTAATATTGT | TTCAGGAAT | GCAACTCTTG | GGGCAATAG | TGCCAAAACG | GATGCTAACG |
| 1921 | GTAAGGCAAC | CGTAACGTTG | AAGTCGAGTA | CGCCAGGACA | GGTCGTCGTG | TCTGCTAAAA |
| 1981 | CCGCGGAGAT | GAGTTCAGCA | CTTAATGCCA | GTGCGGTTAT | ATTTTTTGAT | CAAAACCAAGG |
| 2041 | CCAGCATTAC | TGAGATTAA | GCTGATAAGA | CAACTGCAGT | AGCAAAATGGT | AAGGATGCTA |
| 2101 | TTAAATATAC | TGTAAAAGTT | ATGAAAAACG | GTCAGCCAGT | TAATAATCAA | TCCGTTACAT |
| 2161 | TCTCAACAAA | CTTTGGGATG | TTCAACGGTA | AGTCTCAAAC | GCAAGCAACC | ACGGGAAATG |
| 2221 | ATGGTCGTGC | GACGATAACA | CTAACTTCCA | GTTCCGCGCG | TAAAGCGACT | GTTAGTGCGA |
| 2281 | CAGTCAGTGA | TGGGGCTGAG | GTTAAAGCGA | CTGAGGTCAC | TTTTTTTGAT | GAAGTAAAAA |
| 2341 | TTGACAACAA | GGTTGATATT | ATTGGTAACA | ATGTCAGAGG | CGAGTTGCCT | AATATTTGGC |
| 2401 | TGCAATATGG | TCAGTTTAAA | CTGAAAGCAA | GCGGTGGTGA | TGGTACATAT | TCATGGTATT |
| 2461 | CAGAAAATAC | CAGTATCGCG | ACTGTCGATG | CATCAGGGAA | AGTCACTTTG | AATGGTAAAG |
| 2521 | GCAGTGTGCT | AATTTAAAGC | ACATCTGGTG | ATAAGCAAAC | AGTAAGTTAC | ACTATAAAAG |
| 2581 | CACCGTCGTA | TATGATAAAA | GTGGATAAGC | AAGCCTATTA | TGCTGATGCT | ATGTCCATTT |
| 2641 | GCAAAAATTT | ATTACCATCC | ACACAGACGG | TATTGTCAGA | TATTTATGAC | TCATGGGGGG |
| 2701 | CTGCAAATAA | ATATAGCCAT | TATAGTTCTA | TGAACTCAAT | AACTGCTTGG | ATTAAACAGA |
| 2761 | CATCTAGTGA | GCAGCGTTCT | GGAGTATCAA | GCACTTATAA | CCTAATAACA | CAAAACCCCTC |
| 2821 | TTCTTGGGGT | TAATGTTAAT | ACTCCAAATG | TCTATGCGGT | TTGTGTAGAA | TAATTCCTATA |
| 2881 | ACCACCCCGG | CTAAAATATG | TATTGTTTTA | GTGCGGGCAT | AATTATTTCT | TCTTAAGAAA |
| 2941 | TAACCTCTTA | TAATCAAATC | TACTACTGGT | CTTTTTATCT | GCTTAATAGG | TCTCTTTCAA |
| 3001 | AGAGACACAT | TCACGTTTTC | TAGAGTAGGT | TGATCCAACC | ACGCTGTATA | CCAAAGCTGA |
| 3061 | ATCACATCAA | GCAACAACCTA | TGCTCACAAC | ATCCACACAA | TAAAAA | |

FIG. 4
SUBSTITUTE SHEET (RULE 26)

Sn20-MM2 eae
(3144 bp)

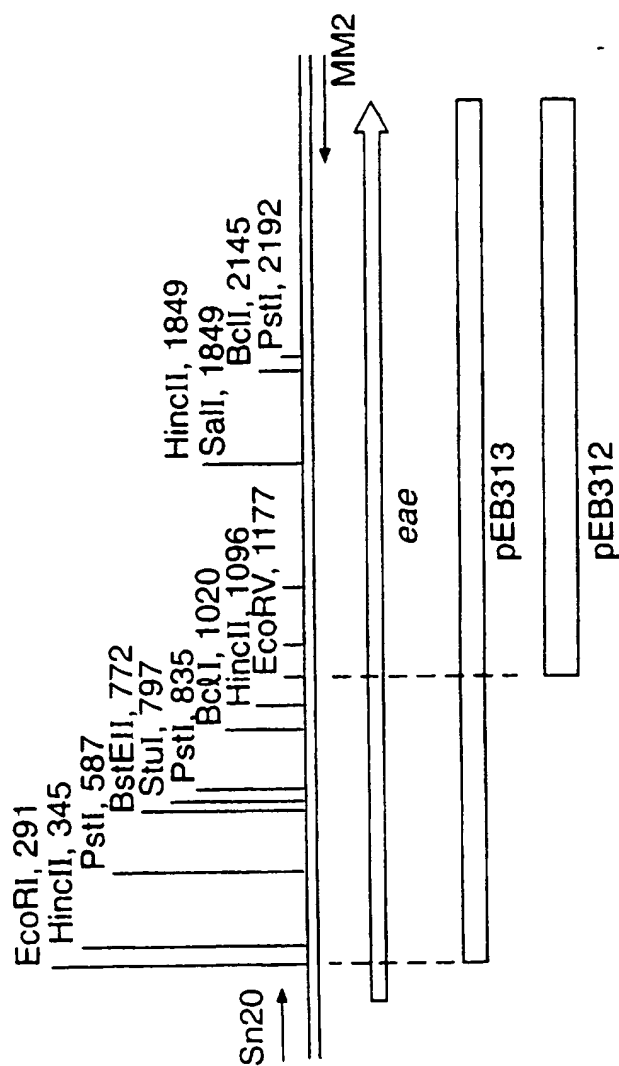
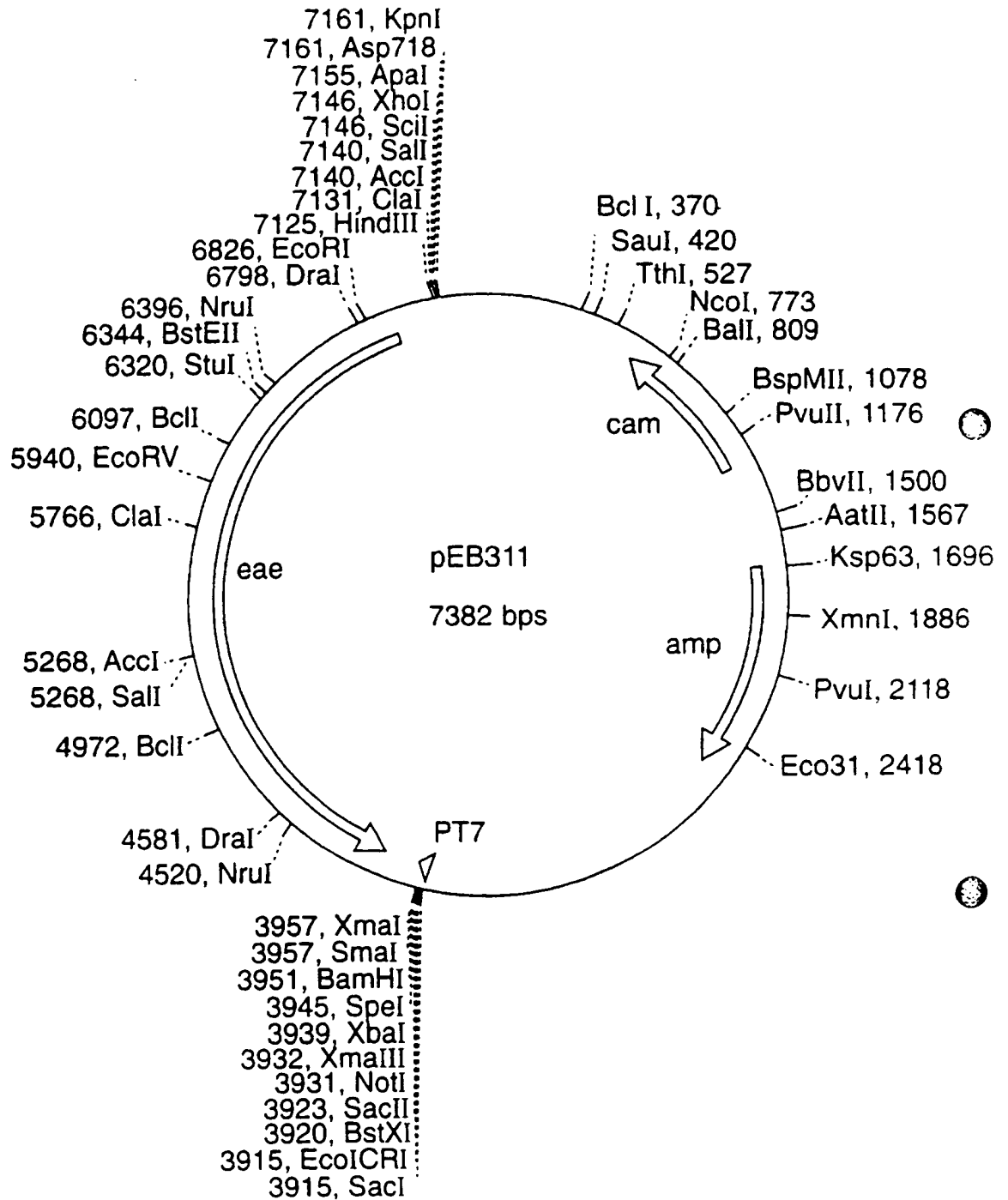
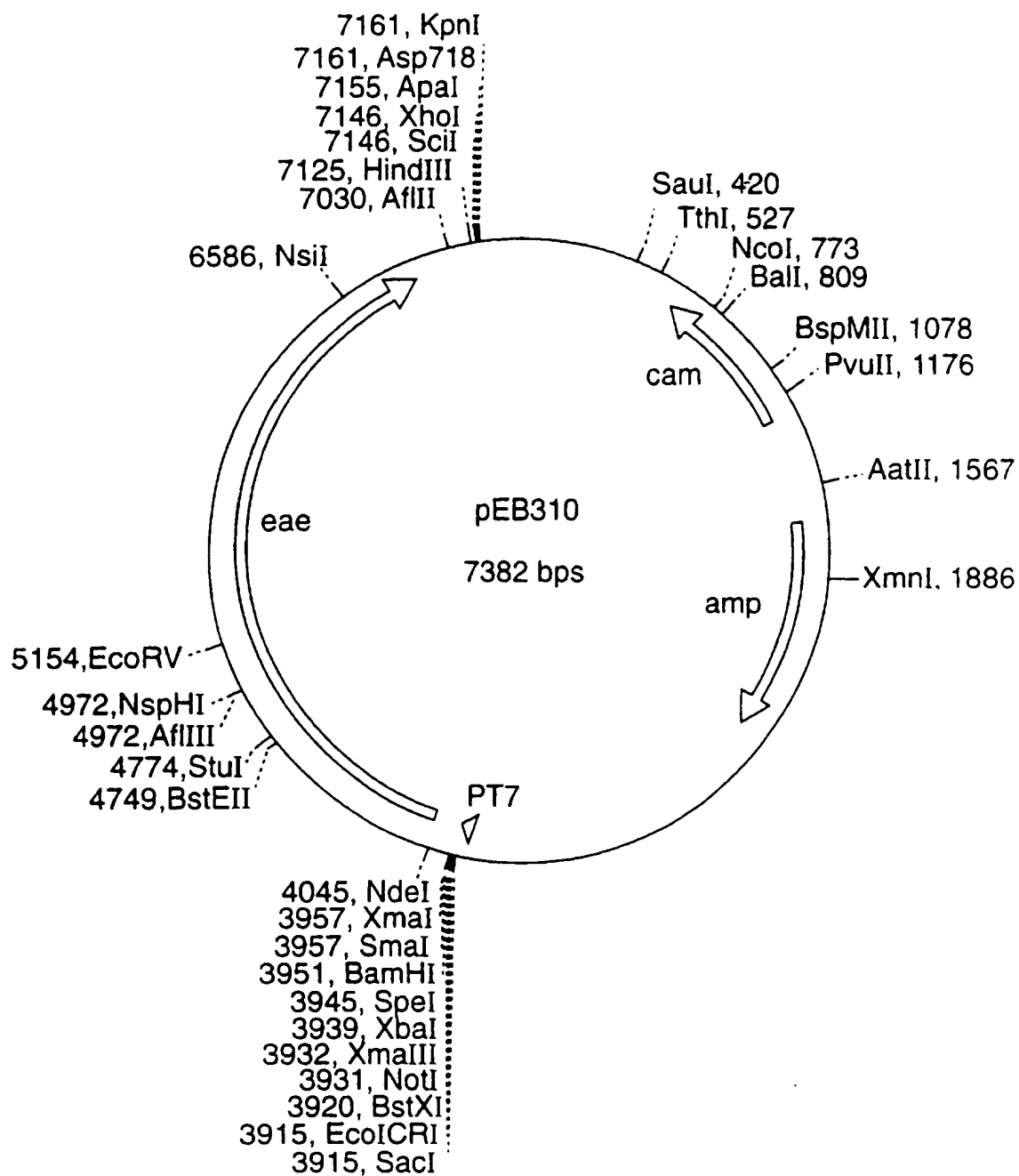


FIG. 5

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**FIG. 6****SUBSTITUTE SHEET (RULE 26)**

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**FIG. 7****SUBSTITUTE SHEET (RULE 26)**

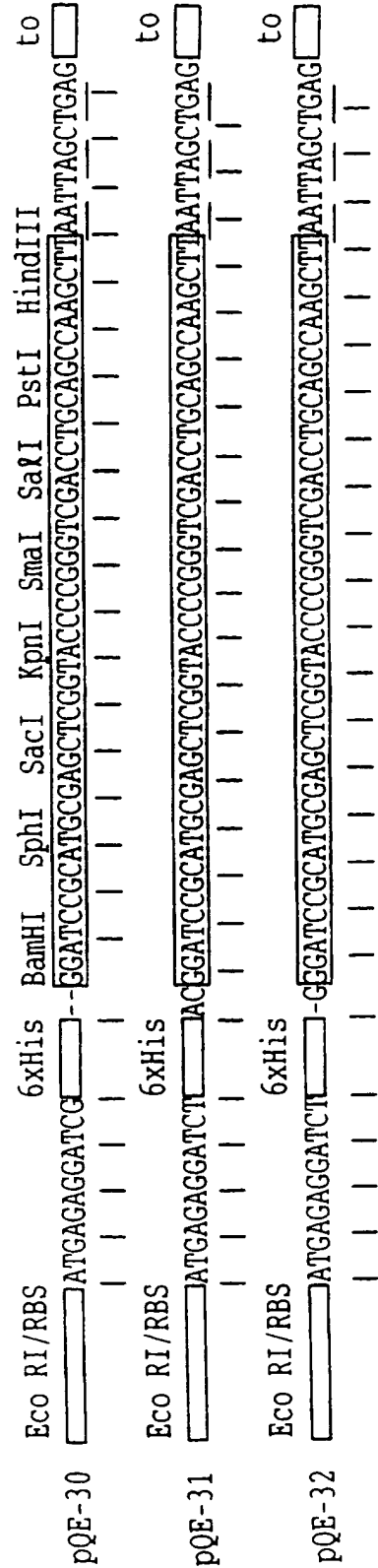
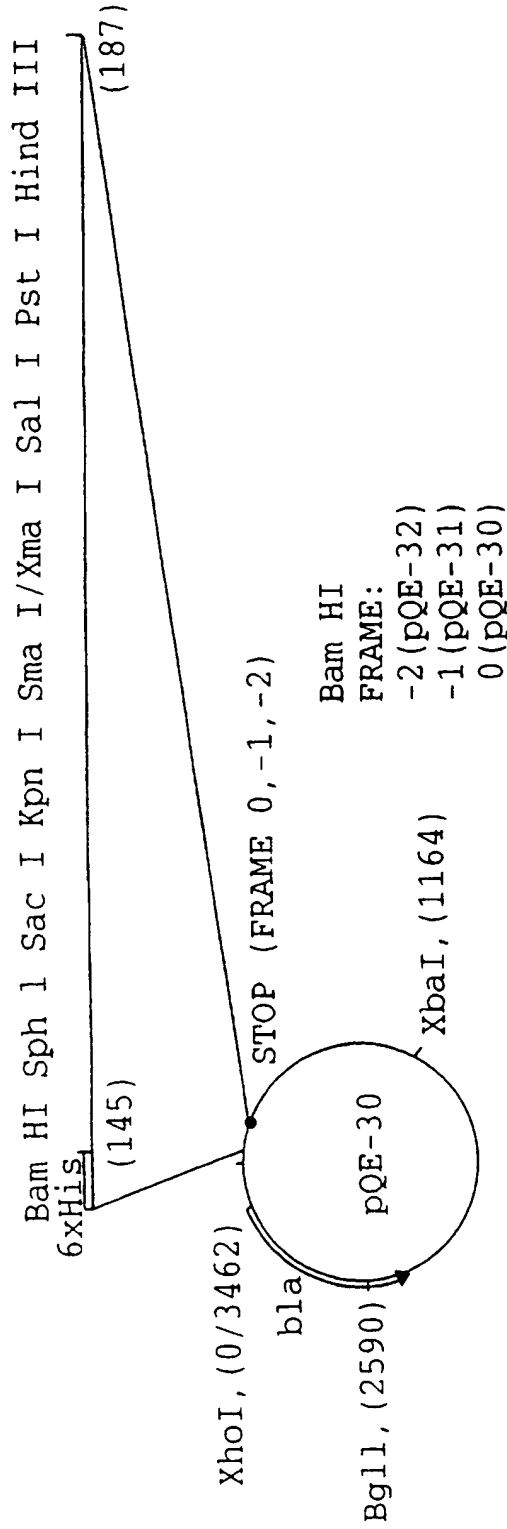
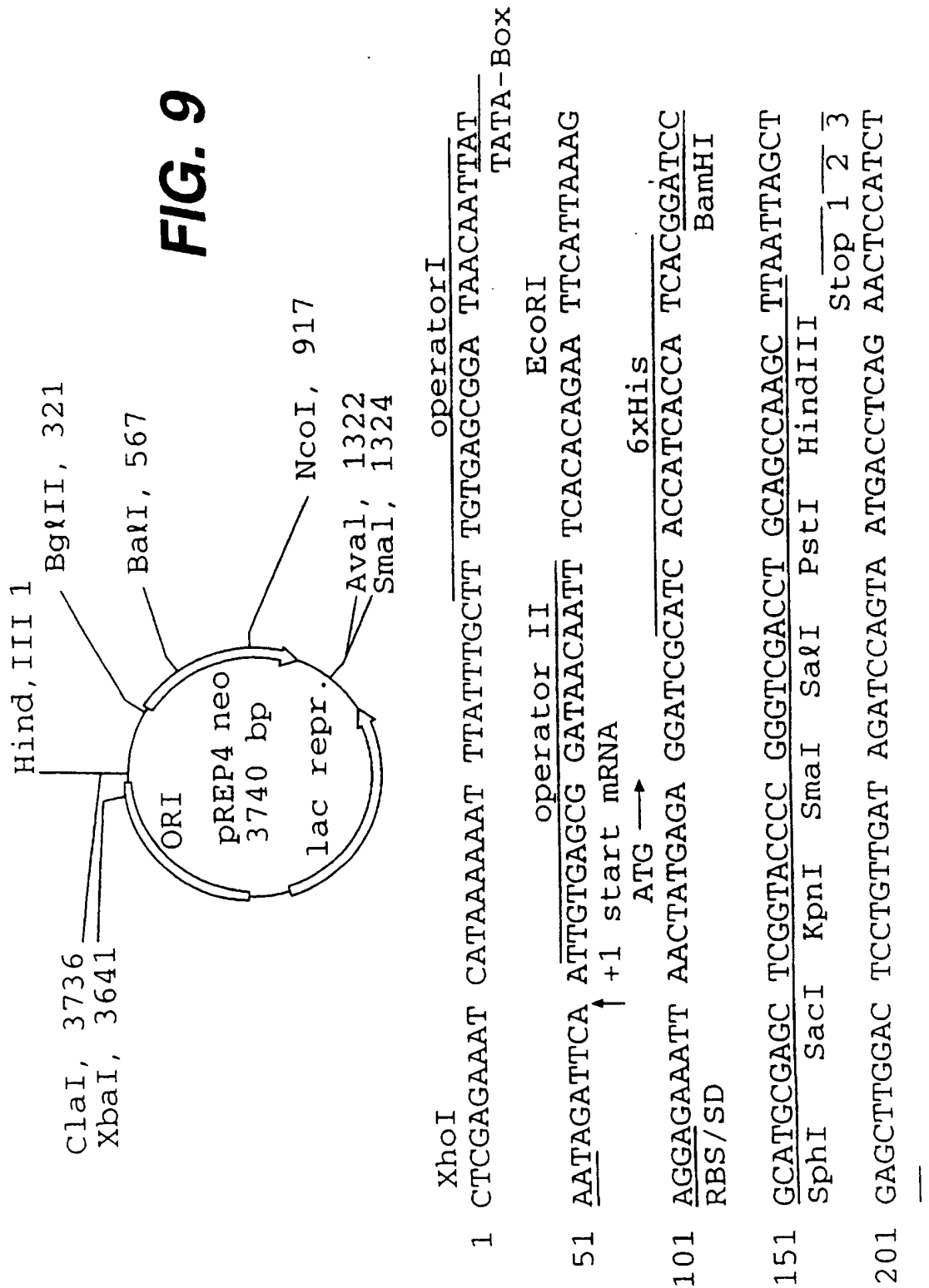


FIG. 8

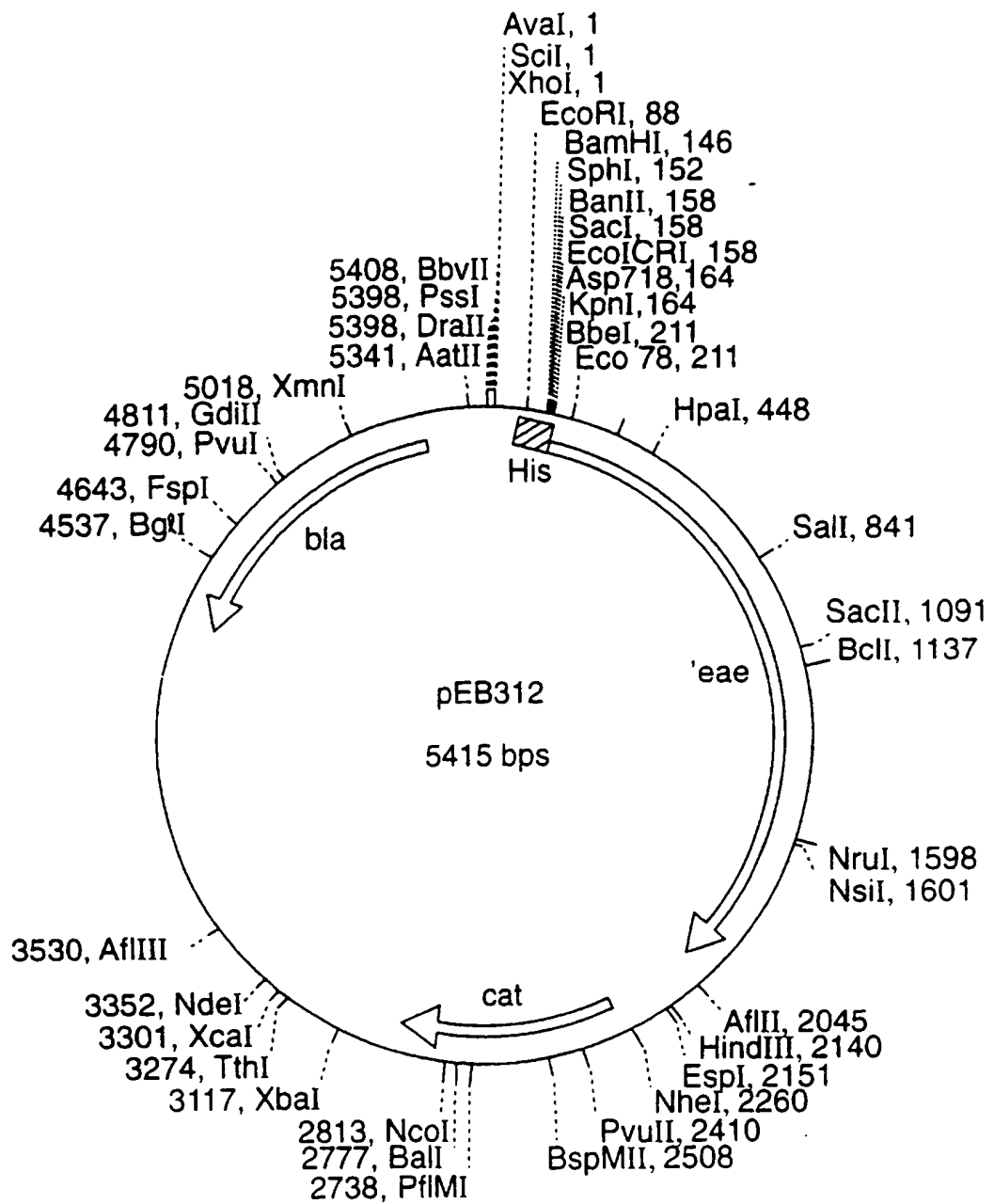
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FIG. 9



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**FIG. 10****SUBSTITUTE SHEET (RULE 26)**

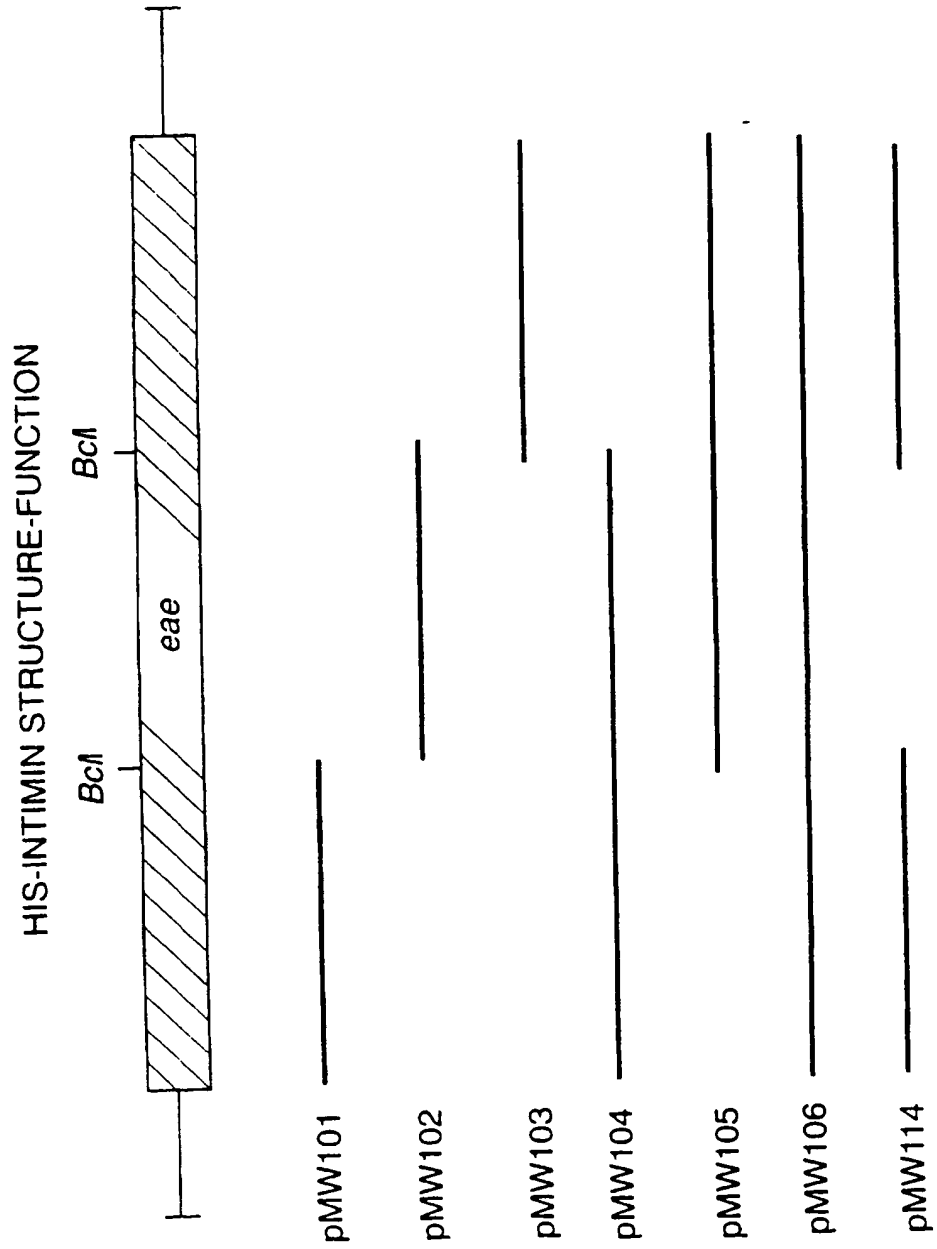


FIG. 11

SUBSTITUTE SHEET (RULE 26)

INTIMIN: C-TERMINAL CONSTRUCTS

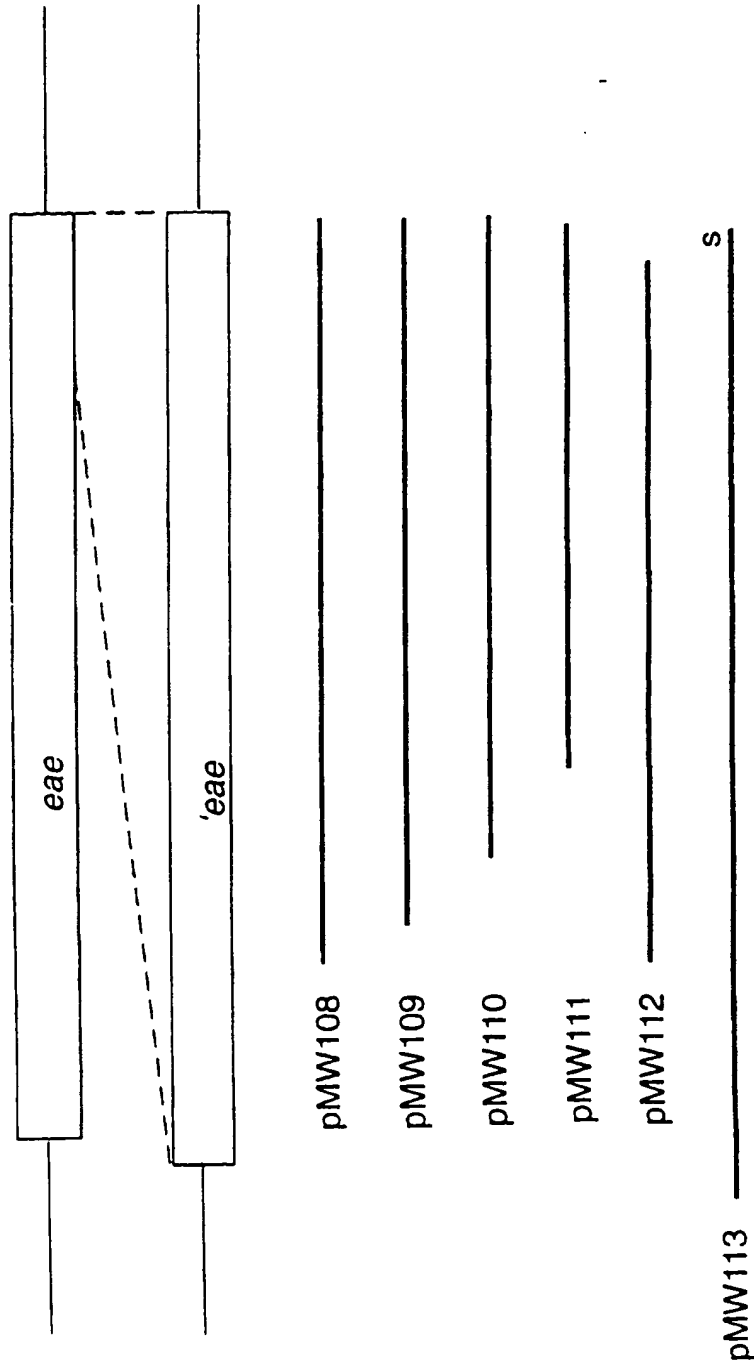
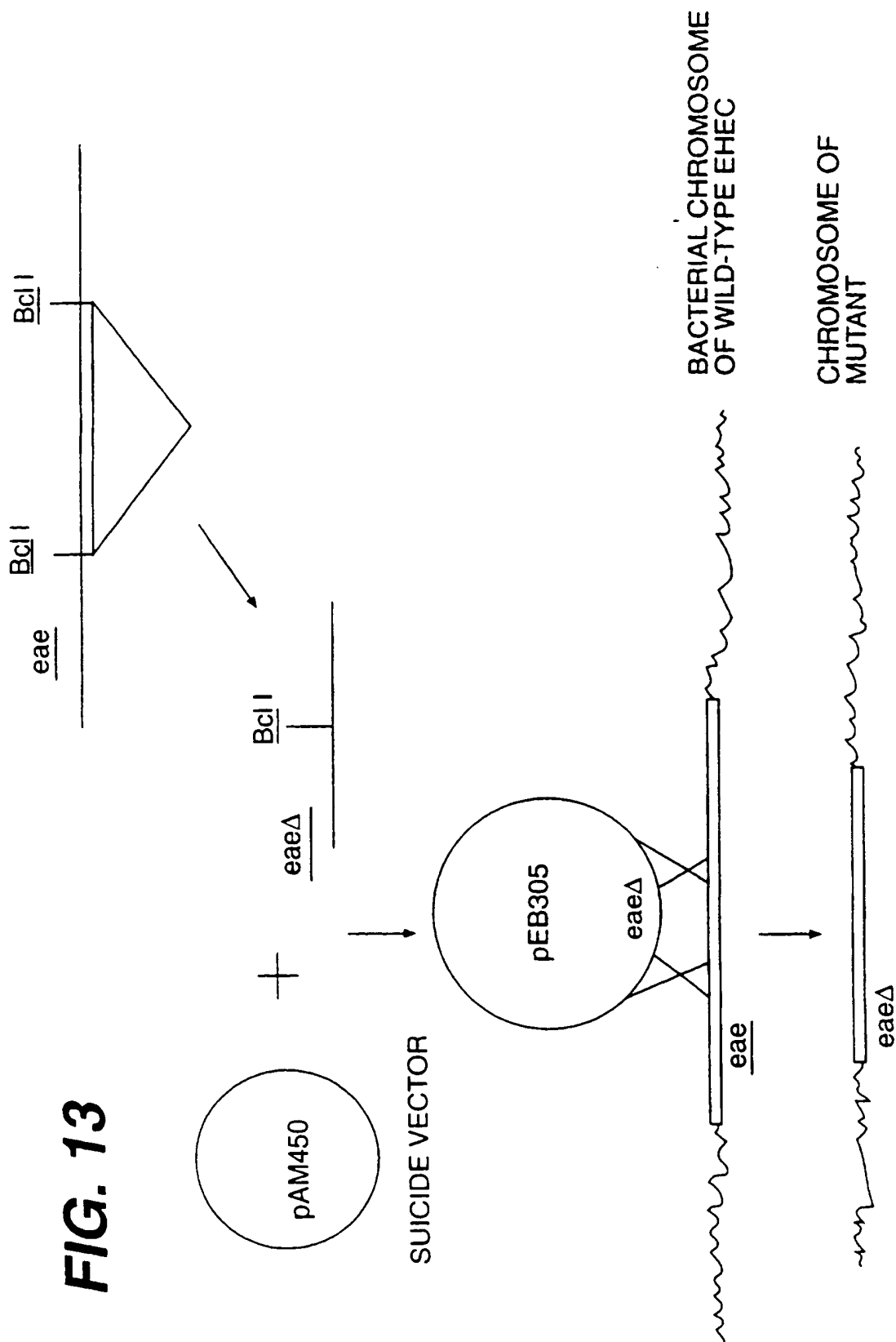


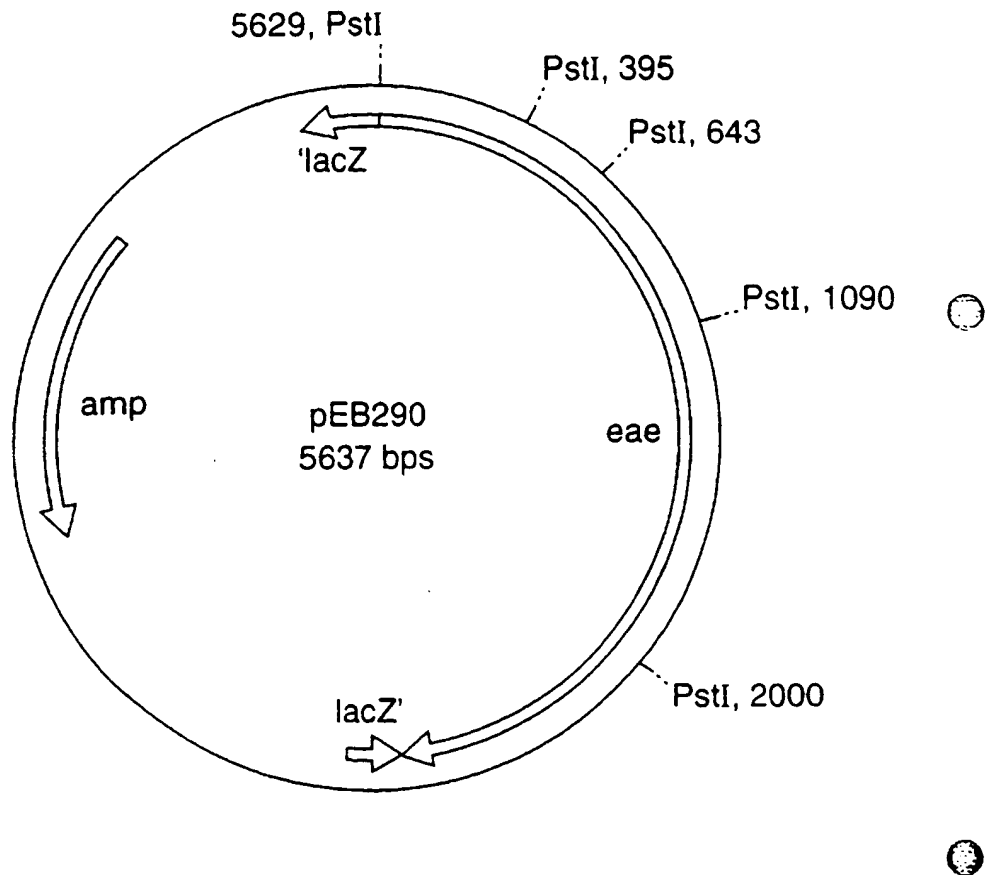
FIG. 12

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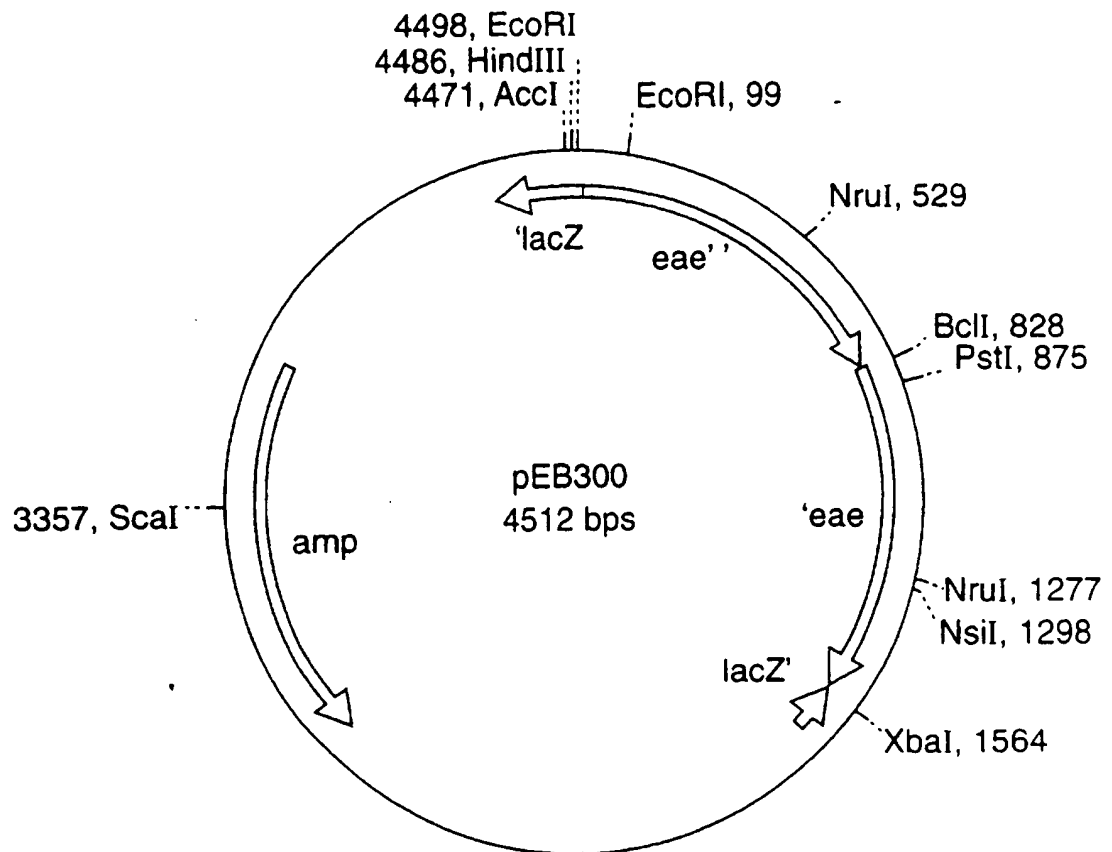


SUBSTITUTE SHEET (RULE 26)

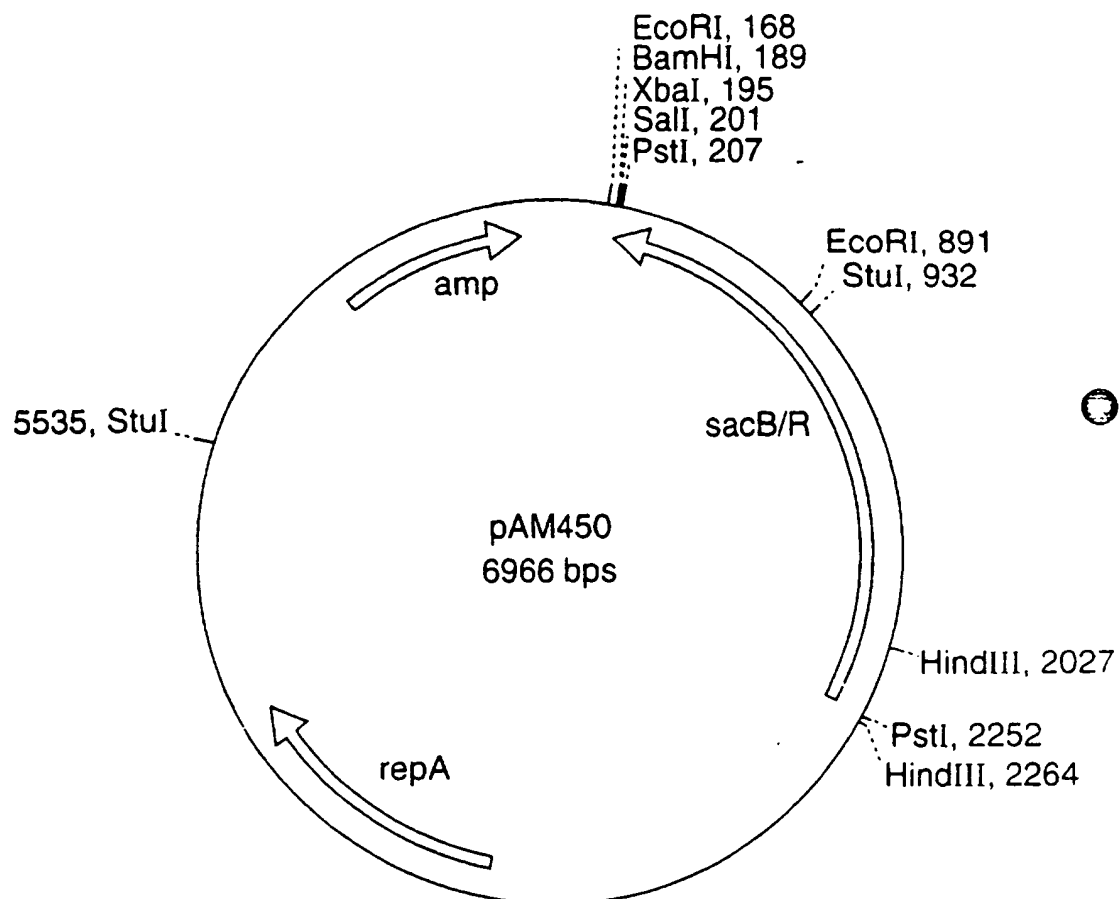
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**FIG. 14****SUBSTITUTE SHEET (RULE 26)**

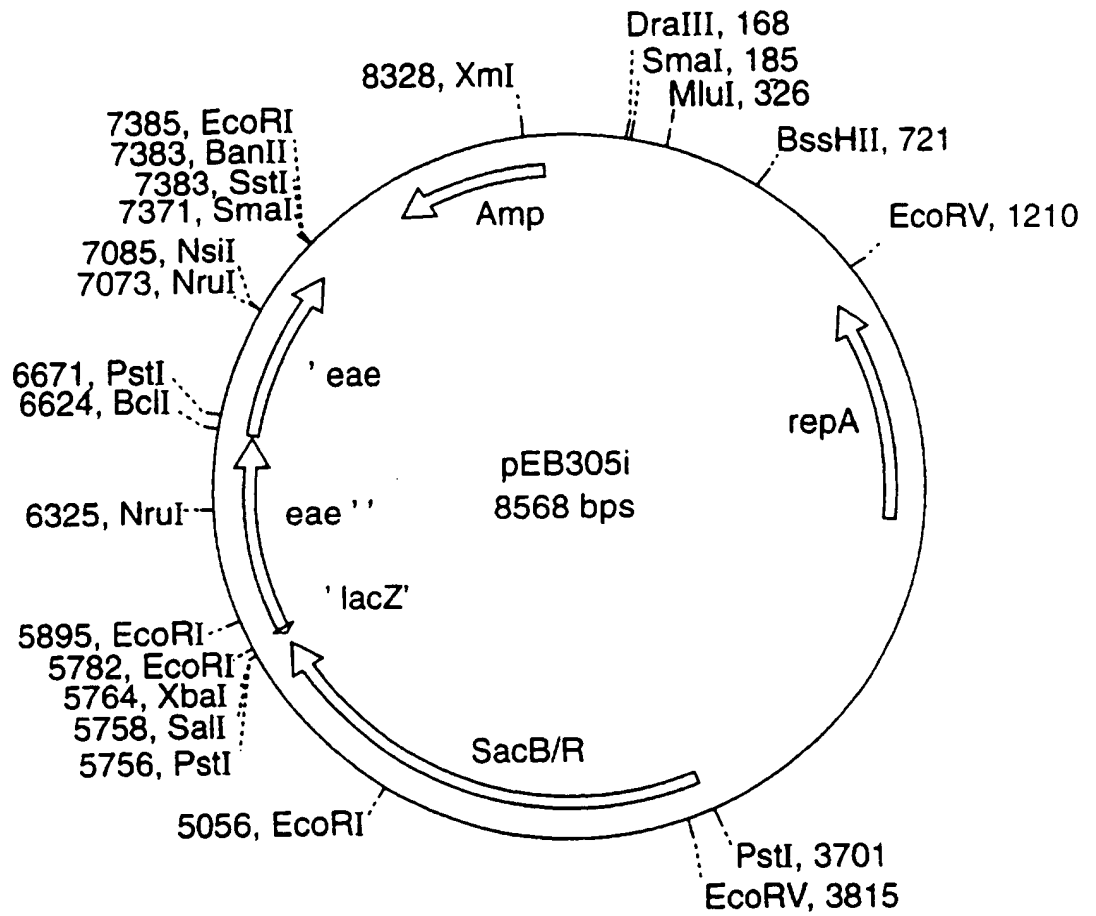
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**FIG. 15****SUBSTITUTE SHEET (RULE 26)**

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**FIG. 16****SUBSTITUTE SHEET (RULE 26)**

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**FIG. 17****SUBSTITUTE SHEET (RULE 26)**

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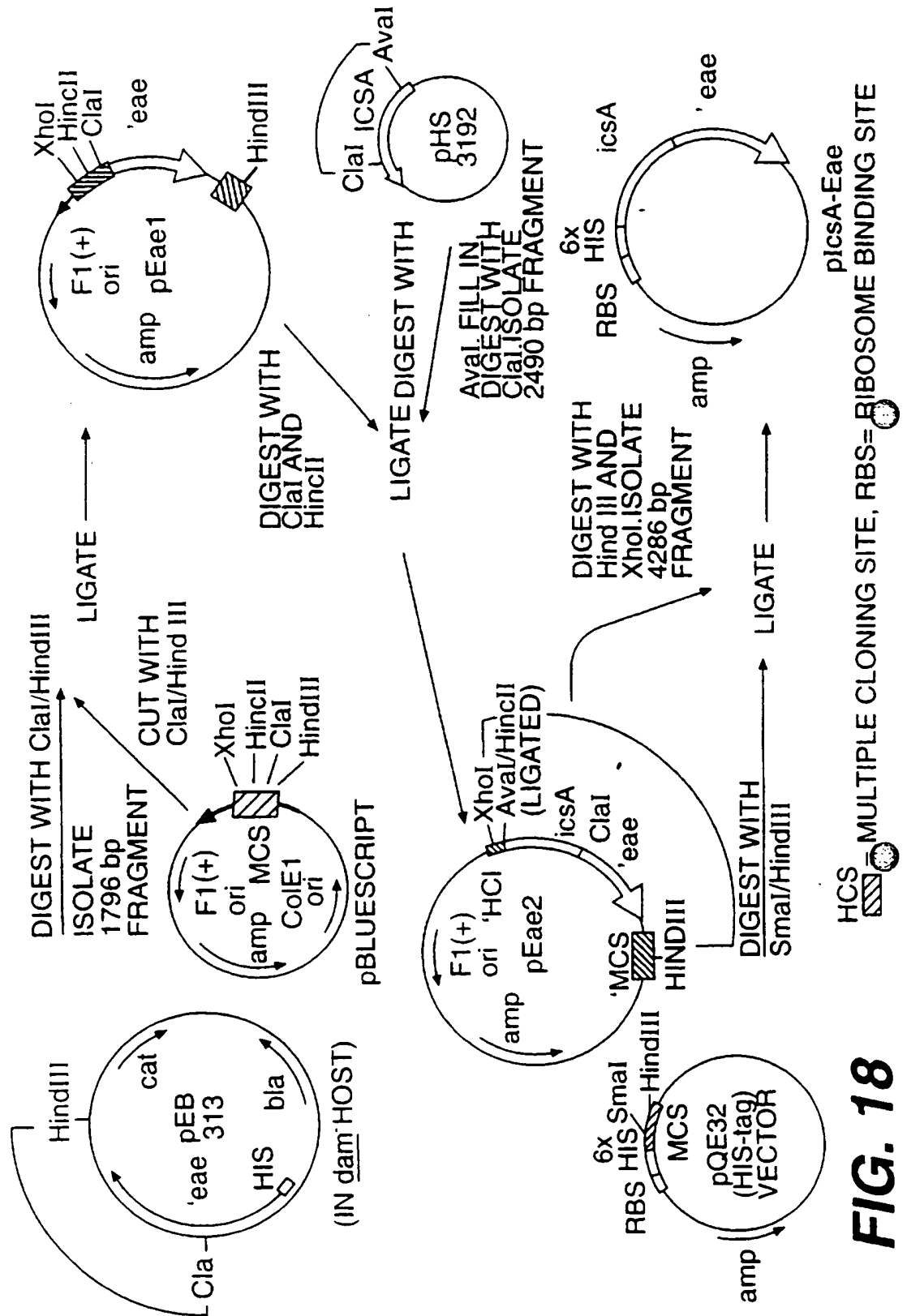


FIG. 18

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 97/05832

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/31 C12N15/62 C07K14/24 C07K14/245 A61K38/16
 A61K39/108 A61K39/02 C07K16/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|----------------------------------|
| X | DATABASE DISSABS AN 95:64682, 1995 MCKEE, M.L.: "Adherence of enterohemorrhagic Escherichia coli to human epithelial cells: the role of intimin (bloody diarrhea, renal failure, hemorrhagic colitis)" XP002040904 | 1-5, 7-14, 28-31, 33,39 |
| Y | see abstract & MCKEE, M.L.: DISSERTATION 1995, AVAILABLE DISSERTATION ABSTRACTS INTERNATIONAL ORDER NO.: AAI9540040, --- | 18,50 |
| | -/- | |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- * "A" document defining the general state of the art which is not considered to be of particular relevance
- * "E" earlier document but published on or after the international filing date
- * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- * "O" document referring to an oral disclosure, use, exhibition or other means
- * "P" document published prior to the international filing date but later than the priority date claimed

- * "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- * "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- * "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * "&" document member of the same patent family

Date of the actual completion of the international search

17 September 1997

Date of mailing of the international search report

30.09.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
 Fax (+31-70) 340-3016

Authorized officer

Maddox, A

INTERNATIONAL SEARCH REPORT

I. International Application No
PCT/US 97/05832

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|--|--|
| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | CA 2 078 716 A (MOUNT SINAI HOSPITAL CORP.) 22 March 1994 | 1,2, 15-17, 28-32, 45-49 18,50 |
| Y | see the whole document | |
| X | FRANKEL, G., ET AL.: "Characterization of the C-terminal domains of intimin-like proteins of enteropathogenic and enterohemorrhagic Escherichia coli, Citrobacter freundii, and Hafnia alvei" INFECTION AND IMMUNITY, vol. 62, no. 5, May 1994, pages 1835-1842, XP002040895 see the whole document | 1,2,6, 45,46 |
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| X | LOUIE, M., ET AL.: "Expression and characterization of the eaeA gene product of Escherichia coli serotype O157:H7" INFECTION AND IMMUNITY, vol. 61, no. 10, October 1993, pages 4085-4092, XP002040897 see the whole document | 1,2,6, 45,46 |
| P,X | MCKEE, M.L., ET AL.: "Truncated enterohemorrhagic Escherichia coli (EHEC) O157:H7 intimin (EaeA) fusion proteins promote adherence of EHEC strains to HEp-2 cells" INFECTION AND IMMUNITY, vol. 64, no. 6, June 1996, pages 2225-2233, XP002040898 see the whole document | 1-5, 7-14, 28-31, 33,39 |
| A | WO 96 00233 A (CHILDREN S HOSPITAL AND MEDICA ;UNIV WASHINGTON (US); TARR PHILLIP) 4 January 1996 see the whole document | 1-50 |
| | -/- | |

INTERNATIONAL SEARCH REPORT

In. International Application No.

PCT/US 97/05832

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | BEEBAKHEE, G., ET AL.: "Cloning and nucleotide sequence of the eae gene homologue from enterohemorrhagic Escherichia coli serotype 0157:H7" FEMS MICROBIOL. LETT., vol. 91, 1992, pages 63-68, XP002040899 see the whole document | 1-50 |
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| A | EP 0 282 042 A (HOFFMANN LA ROCHE) 14 September 1988 see the whole document | 1-50 |
| A | MCKEE, M.L., ET AL.: "Enterohemorrhagic Escherichia coli 0157:H7 requires intimin to colonize the gnotobiotic pig intestine and to adhere to HEp-2 cells" INFECTION AND IMMUNITY, vol. 63, no. 9, September 1995, pages 3739-3744, XP002040902 see the whole document | 1-50 |
| A | JERSE, A.E., ET AL.: "The eae gene of enteropathogenic Escherichia coli encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid" INFECTION AND IMMUNITY, vol. 59, no. 12, December 1991, pages 4302-4309, XP002040903 see the whole document | 33,39 |
| 1 A | WO 92 02820 A (WISCONSIN ALUMNI RES FOUND) 20 February 1992 see the whole document | 28-32 |

Form PCT/ISA-210 (continuation of second sheet) (July 1993)

INTERNATIONAL SEARCH REPORT

international application No.

PCT/US 97/ 05832

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15-27, 33-44 and 47-50 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest
- ☐ No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Patent Application No

PCT/US 97/05832

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | EP 0494293 A | 15-07-92 |
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